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Three variants of western equine encephalomyelitis virus: factors which influence plaque size and the relationship of size to virulence and antigenic properties

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THREE VARIANTS OF WESTERN EQUINE ENCEPHALOMYELITIS VIRUS: FACTORS WHICH INFLUENCE PLAQUE SIZE AND THE RELATIONSHIP OF SIZE TO VIRULENCE AND ANTIGENIC PROPERTIES

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INTRODUCTION

Since the original isolation of western equine encephalomyelitis (WEE) virus by Meyer et al. (39) in 1930, many new laboratory techniques have become available for the detailed study of animal viruses. One of the most significant advancements was the introduction of the cell culture plaque technique by Dulbecco (15), first reported in 1952. A virus population that can be prepared by selecting and propagating the virus harvested from one isolated plaque, theoretically represents the progeny of an individual parent particle. Thus, by employing the plaque technique, the virologist for the first time was able to select variants from among the original population in order to study and compare their individual properties. Significant variations in the characteristics of the newly-derived sub-populations have often been discovered.

Application of selective cloning procedures to populations of WEE virus have resulted in numerous recent reports which reveal the heterogeneity of this virus. These findings have established that many WEE virus isolates are composed of mixtures of subtypes which may vary with respect to plaque diameters in cell cultures, virulence for laboratory animals, or antigenic characteristics.

The subject of WEE virus heterogeneity has been quite extensively investigated by a number of different research groups. However, as the subsequent review of the literature will indicate, some discrepancies appear in the results reported, and some of the following questions could be raised. Are there two, three, or more plaque-size variants of WEE virus? Under what conditions is each type most likely to be found? Is the change in plaque size which one encounters during serial passage due to a true genetic change, or just the result of host selection from among an impure population? And, is it possible that the plaque-size marker is an indicator of variation in other biological properties such as virulence or antigenic characteristics?

The study to be reported here is an investigation of the biological properties of three plaque-size variants of WEE virus which have been cloned from one original parent strain. It was undertaken as an attempt to answer some of these questions, and as an attempt to resolve some of the discrepancies appearing in the literature to be cited.

REVIEW OF THE LITERATURE

Variation in Plaque Diameter

Introduction

One of the few visible clues available to the virologist for use in the identification of an animal virus is its characteristic plaque diameter in cell cultures. Plaque diameter is a readily measurable property of a virus, but unfortunately like many other biological properties, it may be observed to vary. In fact, there are two types of plaque-size variation. One type depends upon the environmental conditions under which plaque diameters are determined. The second type of variation can be attributed to changes in the properties of the virus itself.

The environmental factors responsible for the first type of variation have been quite adequately reviewed by a number of authors (1, 12, 13, 17, 33 and 41). Among those factors mentioned are the species of origin and the type of cell culture in which the virus is titrated. The conditions that are maintained during the incubation of infected cultures, such as the chemical composition of the overlay medium, the temperature, the pH and the time allotted for plaque development also significantly affect plaque size.

However, even under constant and controlled environmental conditions, differences may still be detected in the diameter of the plaques produced by some viruses. This second type of variation, which in the case of WEE virus is associated with the existence of plaque-size variants, was the principal topic of this research project. Therefore, the articles cited in the following review of the literature will be those which directly pertain to this second type of variation in plaque diameter.

WEE virus plaque-size variants

Quersin-Thiry (48) was the first research worker to report the existence of a so-called small-plaque (SP) "mutant" of WEE virus. After 36 hours of incubation in primary chicken embryo fibroblast (CE) monolayer cell cultures, the average SP diameter was only 1.0 mm., in contrast to the 6.0 mm. average diameter of the large-plaque (LP) "wild type".

Three plaque types of WEE virus were described by Marshall et al. (34) in a later publication. When their diameters were measured after 5 days in CE cells, these plaques could be classified as SP (1.3 to 3.0 mm.), medium plaque or MP (approximately 5.0 mm.) and LP (7.6 to 13.0 mm.).

Neither Quersin-Thiry nor Marshall's group discussed the effect of agar overlay composition on the

relative plaque size of WEE virus variants.

Relation of agar overlay components to plaque-size variation of other viruses

In 1960, Nomura and Takemori (44) reported the existence of plaque-size mutants of polio virus. Takemori and Nomura (54) then demonstrated a probable cause for the differences in plaque diameters. They suggested that it was due to a relative difference in the susceptibility of the mutants to the inhibitory effects of a component of Noble agar which was used in the overlay medium.

Similar observations were made by Takemoto and Liebhaber (56) in the case of plaque-size variants of mouse encephalomyocarditis virus. They identified the inhibitory agent as a sulfated polysaccharide, and were able to block its inhibitory activity by incorporating either diethylaminoethyl (DEAE) dextran or protamine sulfate in Noble agar overlay media (55).

After the initial reports cited above, nearly 100 papers soon followed -- all dealing with plaque-size variants of many viruses in relation to agar overlay composition. Only those which pertain directly to the problem being discussed will be reviewed here.

Inhibitors of WEE virus plaque development

The effect of the agar inhibitor on WEE virus was first mentioned in a paper by Ushijima et al. (57). They observed two plaque types which measured 2.0 and 6.0 mm. respectively, after 54 hours under Noble agar overlays on CE cells. Under methyl cellulose, both plaque types were smaller, but the relative size difference was unchanged. When DEAE dextran was added to agar overlays, however, the average SP diameter was increased from 2.0 to 5.0 mm., while the LP diameter remained relatively unchanged. They were led to conclude therefore, that the size difference was primarily due to the greater sensitivity of the SP type to the agar inhibitor.

As a result of work in this laboratory, done independently from that of Ushijima's group, Brown (3) and Brown and Packer (4) also reported plaque-size variants of WEE virus. After 48 hours in CE cell cultures, the variants were classified as SP (2.0 to 5.0 mm.) and LP (7.0 to 10.0 mm.). In this instance, neither the presence of autogenous interferon in inocula, nor autoinhibition by heat-inactivated WEE virus particles were the principal cause of the retarded development of the small plaques. The addition of protamine sulfate to Noble agar overlay medium allowed the small plaque to achieve a diameter equal to that of the large. A difference in the susceptibility of the

variants to the agar inhibitor was therefore suggested as the most likely cause of the plaque-size differences observed.

Using the strain of WEE virus which had been originally studied by Quersin-Thiry, LeClerc and Cogniaux-LeClerc (31) also demonstrated that the presence or absence of interferon is not the most significant factor governing plaque size variation. They found that both the LP and SP variants were equally susceptible to inhibition by interferon, and were equally capable of stimulating its synthesis and release from infected cells. They also reported the increase in diameter of small plaques in the presence of protamine sulfate.

Interactions between arboviruses and agar extract

Colón, Idoine and Brand (10) were able to show that an extract from Noble agar inhibited replication of WEE virus if it was added to the fluid maintenance medium of infected CE cell cultures. Hemagglutination of goose erythrocytes was also inhibited if they were washed with agar extract prior to the addition of virus. These inhibitory effects could be reversed by either DEAE dextran or protamine sulfate.

Colón and Idoine (9) later extended their studies to show the effectiveness of the agar-extract inhibitor

against preparations of infectious ribonucleic acid (RNA) from western (WEE), eastern (EEE) and Venezuelan (VEE) equine encephalomyelitis viruses. The addition of DEAE dextran to agar overlays reportedly caused a three- to four-fold increase in the diameter of the plaques obtained from titrations of infectious RNA on CE monolayers. DEAE dextran speeded the appearance and development of the plaques, but caused no increase in their numbers. Enhancement by DEAE dextran could only be demonstrated with a SP, attenuated type of VEE, and not with a LP type VEE virus which possessed greater virulence for mice.

To date, one of the most detailed studies of the interactions between susceptible arboviruses and the sulfated polysaccharide inhibitor is that reported by Colon et al. (11). They demonstrated a 2-log decrease in titer of EEE virus following incubation with agar extract for 1 hour prior to plating. The interaction between virus and inhibitor occurred over a wide pH range, increased in effect up to at least 1 hour and perhaps to 3 hours of incubation, and was not reversed by dilution of the virus-inhibitor mixture in brain-heart-infusion broth.

Reduced plating efficiency of EEE virus was also observed after treatment of CE monolayers with agar

extract for 1 hour prior to infection. The inhibitory effects could not be completely removed by repeated washings of treated cells with DEAE dextran, and a cell-inhibitor interaction was therefore postulated. Although sodium dextran sulfate mimics the action of agar extract when mixed with susceptible viruses, its effect on cells could be reversed by washing with DEAE dextran. The authors therefore suggested that there was a slight difference in the mode of action of the two inhibitors.

Echovirus variant protected by cysteine

Wallis and Melnick (58) found that the resistance to agar polysaccharide of a LP (Pesascek strain) type 4 echovirus was dependent on the presence in the overlay medium of a 1.0 millimolar concentration of cysteine. The presence of cysteine did not protect the SP (DuToit strain) type 4 echovirus against inhibition. In a basal skim milk overlay medium (devoid of lactalbumin hydrolysate and low in cysteine) the LP virus was actually inhibited to a greater extent than was the SP variant. Apparently a possible protective effect of cysteine for WEE virus has not been investigated.

Natural occurrence and stability of WEE variants

Although most investigators are in agreement concerning the effect of agar polysaccharides on WEE virus

plaque-size variants, conflicting observations are to be found elsewhere in their reports. The degree of heterogeneity in plaque diameter of newly-isolated field strains is one area of disagreement. For example, Marshall et al. (34) indicated that of six field strains they isolated from mosquitoes or birds, all were found to be predominantly the LP variant. In contrast, Ushijima et al. (57) described a mosquito isolate which they considered to be heterogenous because it consisted of a mixture of SP and MP variants.

Another controversial point is the degree of stability of the plaque-size variants during serial passage in several laboratory hosts. Ushijima et al. (57) reported that both the LP and SP variants were stable during serial passages either in CE cell cultures or in the brains of suckling mice. Marshall et al. (34) have stated that serial passage in embryonated chicken eggs was the only method which allowed the LP characteristic of their field strains to be maintained. They observed that the SP variant quickly replaced the LP as the major component of the population after either mouse brain or CE cell culture passages. Brown and Packer (4) also reported that the plaque-size properties of a partially-purified LP population were modified by serial passages in CE cell cultures or in weanling mice.

They, in disagreement with Marshall and his coworkers, found a SP type appearing after passage in chicken embryos. A change in the opposite direction has been reported by Quersin-Thiry (48). According to this author, a change in the characteristic plaque size of a population from SP to LP during propagation in CE cell cultures was enhanced by the presence of lactalbumin hydrolysate, yeast extract and bovine serum in the maintenance medium.

Variation in Virulence

Comparative virulence of WEE plaque-size variants

Of those workers who have reported the existence of WEE plaque-size variants, only Quersin-Thiry (48) has attempted to determine their relative virulence, and in that case, only for chicken embryos. No significant differences in virulence between the LP and SP variants could be found.

Comparative virulence of plaque-size variants of other arboviruses

Hardy and Hearn (19) have indicated that a SP variant of Venezuelan equine encephalomyelitis (VEE) virus causes death of weanling mice only by intracranial infection, and not the intraperitoneal route.

In contrast, the LP variant (coincidentally, less susceptible to agar polysaccharide inhibition) is fully virulent by both routes. VEE virus has been classified into three plaque types by Brown (2). All three types were fully virulent for hamsters, but only the LP variant killed mice when injected intraperitoneally. Plaque-size variations of VEE virus, like WEE, were host-dependent, according to Heydrick et al. (23). The latter group has demonstrated a difference in banding patterns in sucrose density gradients, a difference in the ratio of soluble to insoluble lipids and a difference in virulence between VEE variants.

St. Louis encephalitis (SLE) virus exhibits plaque-size heterogeneity also, as observed by Nagai et al. (42). They concluded that the LP and SP variants of SLE virus were separate genotypes which did differ in virulence for mice and in cytopathogenicity for hamster kidney cell cultures, but which were closely related or identical in antigenic properties. Once again, the LP type was the more virulent of the two.

Another example of a correlation between virulence and plaque size is from the work of Porterfield (47). He observed that the neurotropic strains of yellow fever virus produced larger plaques in CE cells, than did the attenuated, chicken-embryo-adapted 17D strain.

Although Mayer (35, 36, 37 and 38) discussed essentially similar findings resulting from studies of Central European tick-borne encephalitis virus, his reports differed significantly in one respect. Use of inhibitor-free agar, or addition of protamine sulfate to the overlays failed to alter the relative diameter of the SP, avirulent variant of this virus.

The work of Inoué and Kato (24) showed that a SP, thermo-efficient mutant of Japanese B encephalitis virus was more virulent for mice, by intranasal inoculation, than was the LP type of the same strain. Neither exhibited pathogenicity when administered to mice subcutaneously or intraperitoneally. In this final example, virulence was inversely proportional to plaque diameter.

Changes in virulence of WEE virus with serial passage

The virulence of WEE virus for baby chicks was investigated by Chamberlain et al. (5) in 1954. They observed that freshly isolated field strains of the virus routinely killed ½-day-old chicks quickly and at high dilution after subcutaneous injection. The Flemming strain of WEE virus, which had undergone many serial intracranial passages in mice, required a much longer time interval to kill, produced more distinctly observable signs of central nervous system disorder in the chicks, and was significantly lower titered.

As Kissling (30) reported in 1957, "Most arboviruses, after continued intracranial passage, lose peripheral virulence." He cited WEE, EEE, SLE, West Nile and Japanese B encephalitis viruses as typical examples.

WEE virus heterogeneity with respect to virulence

When Dunayevitch et al. (18) titrated a field strain of WEE virus in young adult mice, they observed auto-inhibition effects at the lower dilutions. They were subsequently able to clone a variant from the population which exhibited markedly lower virulence for the mice. They reasoned that the mice, inoculated at the lower dilutions, had been protected against the virulent virus by the non-pathogenic variant which coexisted with it in the mixed population of bird origin.

Serial cloning of this non-pathogenic WEE virus was continued by Johnson (25), who selected at each passage level for variants of low virulence for infant mice. The clone 15 variant which resulted was very low in pathogenicity for adult mice by intracranial or by intraperitoneal routes, yet it conferred a strong immunity to subsequent challenge with neurotropic WEE virus. Recent studies by Kemp and Johnson (29) and by Roca-Garcia et al. (51) indicate that the clone 15 variant shows some promise of becoming a satisfactory live-virus vaccine against encephalomyelitis in horses

and perhaps man.

Plaque diameter of the clone 15 variant

Johnson[a] has stated that newly-isolated strains of WEE virus usually produce large (8 to 15 mm.) plaques in duck embryo cell cultures. In contrast, the original bird isolate, from which the clone 15 variant was eventually derived, initially caused the formation of intermediate-sized plaques (3 to 4 mm.) in duck embryo cell cultures, and a mixture of small and intermediate-sized plaques (actual measurements not specified) in CE cell cultures. Although plaque properties were not considered in the selection of this variant, it is of interest that, even in its wild state, the plaques produced by the parent population differed from those of the typical isolate.

Variation in Antigenic Properties

Antigenic properties of WEE plaque-size variants

Investigators of WEE plaque-size variants have routinely been unsuccessful in attempts to distinguish

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[a]Johnson, H. N., The Rockefeller Foundation, Berkeley, California. Unpublished observations. Private communication, 1965.

between them antigenically. Quersin-Thiry (48), for example, observed that the LP and SP variants were neutralized to the same extent by antiserum from rabbits immunized with killed LP virus. The two variants studied by Ushijima et al. (57) could not be distinguished serologically by neutralization tests employing either chicken antiserum or a standard reference antiserum. According to Marshall and his coworkers (34), the SP and LP types were antigenically the same in cross-neutralization tests with rabbit antisera. Despite the reported similarities, Marshall's group did observe some apparent differences between the variants. The average plaque diameter of the LP variant, in contrast to the others, was more markedly reduced in the presence of specific antiserum. They also reported that the SP variant appeared to be more effectively neutralized by either homologous or heterologous antisera, than was the LP variant.

Antigenic variation with serial passage

Olitsky et al. (45) asserted that frequent and consecutive intracranial passages in some unknown manner caused their stock strain of WEE virus to undergo an immunogenic change during the years that it was maintained at the Rockefeller Institute. They found that mice which had been immunized with several newly isolated

strains of WEE virus were unprotected against challenge by their stock strain, if a high passage level of this strain was used for challenge. The mice were immune to challenge however, with an equivalent dosage of the same stock strain if a lower passage level was used as the challenging virus.

Using neutralization, complement-fixation and hemagglutination-inhibition tests, Karabatsos, Bourke and Henderson (28) also noted significant differences between newly-isolated strains and older laboratory strains of WEE virus. Since these antigenic differences were so great, they avoided classification of the isolates as new arboviruses, only after selective cloning procedures demonstrated an interesting degree of antigenic heterogeneity in both the old and new strains.

Antigenic heterogeneity

Henderson (21), by selective cloning of ten WEE strains, found that most contained sub-populations having antigenically distinct properties. He suggested that the quantitative distribution of common but distinct antigens determined the immunological characteristics of each strain. Since they existed as mixed populations, the antigenic properties of these strains could be modified by host selection during serial passage. This type of host selection may markedly alter the results

of serological tests used to determine WEE virus antigenic relationships, according to Saturno and Henderson (52). In their latest reported study, Henderson, Shah and Wallis (22) detected differences in the replication rates and differences in the rates of release of two antigenic subtypes from CE cells in culture. They suggested that these rate differences might well account for the host-selection phenomenon observed in these cells.

Henderson[a] recently stated, "We have not noted any consistent variation in plaque size during the derivation of distinct plaque antigens from various WEE strains. However, certain unpurified strains do, in our hands, consistently produce smaller plaques than others. Most certainly a correlation between plaque size and antigenic property would be important to pursue...".

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[a]Henderson, J. R., Yale University School of Medicine, New Haven, Connecticut. Unpublished observations. Private communication. 1965.

EXPERIMENTAL PROCEDURE

Source of Viruses

Records strain

This research project was mainly an investigation of the properties of three plaque-size variants of the Records strain of WEE virus. This strain was originally one of seven isolates obtained by Dr. Edward Records during an outbreak of encephalomyelitis in horses and mules in Churchill County, Nevada, in the late summer of 1931. The original laboratory passage was in guinea pigs inoculated intracerebrally with emulsified horse brain tissues. Between 1935 and 1951, the Records strain underwent an indeterminant number of chicken embryo passages at Fort Dodge Laboratories, Fort Dodge, Iowa. It was first obtained by Dr. R. A. Packer of the Department of Veterinary Hygiene, in May of 1951.

Derivation of the plaque-size variants

The sole criterion employed for the selective cloning of three variants from the Records strain was their difference in plaque diameter on CE cell cultures. The cloning experiments and the passage histories, which differed for each of the variants, are outlined in Tables 1, 2 and 3.

Table 1. Derivation of the LP variant

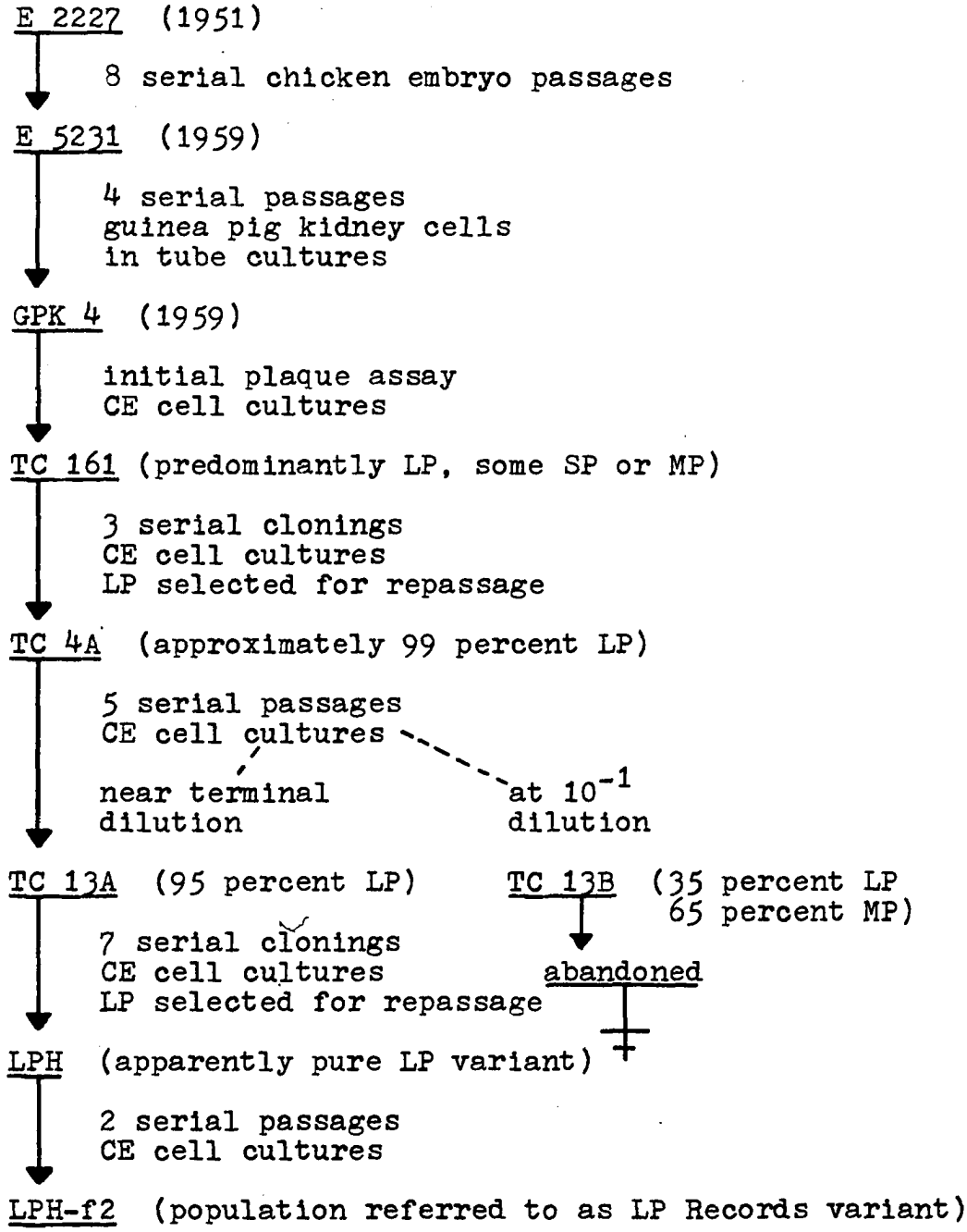

Table 2. Derivation of the MP variant

Records strain obtained from Fort Dodge Laboratories

E 2227 (1951)

↓ 8 serial passages
chicken embryos
low dilution

E 5231 (1959)

↓ 8 additional serial passages
chicken embryos
near terminal dilution

E 5758 (1961)

↓ 4 serial passages
weanling mice
intracranial route
dilutions unrecorded

4 E-M (1961)

↓ 4 additional serial passages
weanling mice
intracranial route
all at low dilution (10^{-1})

11 M first plaqued on CE cells (65 percent MP variant)
(35 percent SP variant)

↓ 1 passage
weanling mice
10^{-4} dilution

11 M-2 replaqued on CE cells (nearly 100 percent MP variant)

↓ 4 serial clonings
CE cell cultures
MP selected for repassage

MP 4A (apparently pure MP variant)

↓ 2 serial passages
CE cell cultures

MP 4A-f2 (population referred to as MP Records variant)

Table 3. Derivation of the SP variant

Records strain obtained from Fort Dodge Laboratories

E 2227 (1951)

↓ 8 serial passages
chicken embryos
low dilution

E 5231 (1959)

↓ 8 additional serial passages
chicken embryos
near terminal dilution

E 5758 (1961)

↓ 4 serial passages
weanling mice
intracranial route
dilutions unrecorded

4 E-M (1961)

↓ 4 additional serial passages
weanling mice
intracranial route
all at low dilution (10^{-1})

11 M first plaqued on CE cells (65 percent MP variant)
(35 percent SP variant)

↓ 4 serial clonings
CE cell cultures
SP selected for repassage

SPN (approximately 2 percent MP impurity remained)

↓ 3 additional serial clonings
CE cell cultures
SP selected for repassage

SPR (no detectable MP impurity)

↓ 1 passage
CE cell cultures, fluid maintenance

SPV (approximately 2.0 percent MP impurity detected)

Recent WEE isolates

Six first-passage WEE virus isolates were obtained through the courtesy of Dr. C. S. Mollohan, Colorado State Department of Public Health, and Dr. Louis C. LaMotte and Mr. Larry Kirk of the Virus Investigations Unit, Disease Ecology Section, U.S. Public Health Service in Greeley, Colorado. All six samples were received in the form of frozen brain suspensions from suckling mice.

One additional isolate was kindly supplied by Robert H. Kokernot, M.D., Assistant Director of the Center for Zoonoses Research, University of Illinois, Urbana, Illinois. The sample received was in its second mouse brain passage, after 2 initial passages in chicken embryos. Table 4 contains a description of the source and date of isolation of each virus.

Cell Culture Techniques

Routine culture procedures

In an earlier paper, the author (3) has described CE cell culture techniques in detail. With the one following exception, identical procedures were used in the present study. In previous work, complete cell culture media were prepared, and then filter sterilized. In this project, cell culture media were autoclaved prior to the addition of sterile antibiotics, sodium

Table 4. Description of seven recent WEE virus isolates

Identification number	Original host	Specimen collected	Geographical location	Date of isolation
65V-241	*Passer domesticus*	whole blood	Texas	July 9, 1965
65V-796	*Passer domesticus*	whole blood	Texas	August 4, 1965
65V-657	*Culex tarsalis*	10 mosquitoes	Texas	July 21, 1965
65V-692	*Culex tarsalis*	10 mosquitoes	Texas	July 24, 1965
65V-633	*Culex tarsalis*	50 mosquitoes	Colorado	July 22, 1965
65V-629	*Culex tarsalis*	50 mosquitoes	Colorado	July 18, 1965
E 2329	3-year-old pony	brain	Illinois	Sept. 11, 1964

bicarbonate and bovine serum. Filtration was used only for the sterilization of trypsin solutions and serum.

The standard plaque assay

Unless otherwise specified, all plaque titrations were performed under the standard plaque assay conditions. They have also been completely described (3), but a brief summary follows. The CE cell cultures were routinely infected at 48 hours of age. Viruses were allowed a period of one hour for adsorption to cells before overlays were added. The overlay medium consisted of Earle's balanced salt solution, adjusted to an initial pH of 7.6 with sodium bicarbonate. The overlays also contained 0.5 percent lactalbumin hydrolysate[a], 5.0 percent bovine serum and 0.75 percent Noble agar[b]. Penicillin (100 units per ml.) and streptomycin sulfate (100 micrograms per ml.) were included. The monolayers were stained with neutral red in a second agar overlay. Plaques were routinely counted and measured 48 hours after cell culture infection.

It should be emphasized that the term "standard" plaque assay", as it is used throughout the remainder of this paper, refers exactly to the entire set of

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[a,b]Difco Laboratories, Inc., Detroit 1, Michigan.

conditions just specified. Because these environmental conditions were maintained as constant as possible in all experiments, any differences in plaque diameters detected were the result of the different properties of the three variants, or the result of selected and controlled changes in the environment.

Fluid media and diluent

For routine virus propagation in CE cell cultures, a fluid maintenance medium was employed. This medium was identical to that used in agar overlays except for the omission of Noble agar. In certain instances to be noted later, the bovine serum was also omitted from the fluid maintenance medium. This same medium, without serum, was also used as a cell culture wash medium, and as the diluent for serum or virus. Dilution blanks were dispensed from freshly prepared medium in the day they were used in order to avoid changes in pH which sometimes occurred with storage. In all the titrations reported, separate pipettes were used at each dilution.

Plaque Diameter Comparisons

The three Records variants

During the course of many experiments, there were ample opportunities to determine the characteristic plaque diameters of the LP, MP and SP Records variants

under standard assay conditions. The relative plaque size of a virus population remained constant. As a result, if a sample of each variant was included in each trial, the plaques obtained provided an excellent standard of comparison and an indicator of the performance of the cell culture system. The degree of purity and the stability of the variant populations could thus be determined. If any changes in the plaque-size properties of a virus population occurred or could be induced, they were readily detectable.

Seven recent WEE virus isolates

Parallel titrations of seven newly-isolated field strains of WEE virus, and of the three Records variants were made on cultures grown from the same lot of CE cells. The plaque-size properties of each virus population were compared using the standard plaque assay procedure.

Effects of agarose overlays

According to DeMaeyer and Schonne (14), agar is composed of two polysaccharides, agaropectin and agarose. It is the agaropectin component which contains most of the sulfate groups normally found in agar. Following the method of Porath and Hjerten (46), an agarose preparation nearly free of negatively-charged ionizable groups can be obtained from Noble

agar. Marine Colloid, Inc., manufactures an agarose[a] with less than 0.1 percent sulfate content. Preliminary experiments with this preparation indicated that a 0.375 percent concentration of agarose yielded overlays of approximately the same consistency as Noble agar overlays at the standard 0.75 percent concentration.

To determine the relative plaque diameters of each Records variant in the virtual absence of sulfate inhibitor, plaques were compared by the following procedure. Duplicate sets of cultures from the same lot of CE cells were inoculated with identical samples of the LP, MP or SP variant. One set received the standard overlay. The duplicate set was overlayed with a medium in which agarose (0.375 percent) had replaced the Noble agar. Diameters of the resulting plaques were compared.

Effects of inhibitors added to agarose overlays

Since agarose overlays are relatively free of inhibitors of WEE virus plaque development, their use provided the opportunity to directly observe the effects on plaque development of known inhibitory substances. Before incorporation in agarose overlay medium, several additives were prepared in the following way.

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[a]Seakem agarose, distributed by Bausch & Lomb, Rochester, New York.

Suspensions of agarose (0.375 percent), Noble agar (0.75 percent) and Bacto agar[a] (1.0 percent) in distilled, deionized water were autoclaved, allowed to cool and solidify in the form of shallow blocks, and then were frozen for 24 hours. After the blocks were thawed, the fluid which escaped was filtered through sterile gauze and centrifuged for 10 minutes at 1,000 rpm. Each of the resulting supernatant fluids (agar extracts) was stored at 5° C. until used. A stock solution of sodium dextran sulfate 500[b] was prepared immediately before use by dissolving 200 mg. in 100 ml. of sterile, deionized water.

As before, different sets of CE cultures grown from the same lot of cells were infected with identical sample inocula of the LP, MP or SP variant. As shown in Table 5, only the overlay composition within each set was varied, and plaque diameter comparisons were otherwise made by the standard assay procedure.

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[a]Difco Laboratories, Inc., Detroit 1, Michigan.

[b]Pharmacia, Uppsala, Sweden.

Table 5. Variations in overlay composition

Basal medium	Additive	Final concentration
Agarose overlay	Agarose extract	5.0 percent
Agarose overlay	Noble agar extract	5.0 percent
Agarose overlay	Bacto agar extract	5.0 percent
Agarose overlay	Dextran sulfate	100 mcg/ml.

Effects of cysteine and lactalbumin hydrolysate in skim milk overlays

To determine the effect on plaque diameters of added cysteine or lactalbumin hydrolysate, it was necessary to first employ a basal medium containing a minimal amount of these ingredients. A skim milk medium has been proposed for this purpose by Wallis and Melnick (58). The medium used in the present study consisted of Earle's balanced salt solution, the usual concentrations of antibiotics, 0.1 percent skim milk and 0.75 percent Noble agar. It was buffered to an initial pH of 7.6 with sodium bicarbonate. The skim milk was added from a 2.0 percent stock solution of instant nonfat dry milk[a] prepared according to the method Rhim and Melnick (50).

[a]Carnation Co., Los Angeles, California.

In parallel assays on cultures from the same lot of CE cells, three variations of the basal medium were also tested. In one set of cultures, the skim milk medium contained L (+) cysteine monohydrochloride[a] in 2.0 millimolar concentration, added immediately prior to use. In a second set, 0.5 percent lactalbumin hydrolysate was added. The third variation was the substitution of 0.375 percent agarose for Noble agar. Plaque diameters of the Records variants under each type of overlay were determined.

Effects of bovine serum

Control media for this experiment were Noble agar and agarose overlays which contained no serum. Test media were the same preparations, but with bovine serum[b] added at a 5.0 percent concentration. The effects of the added serum on the plaque development of each Records variant were thus determined in the two types of overlay medium.

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[a]Pfanstiehl Laboratories, Inc., Waukegan, Illinois.

[b]Grand Island Biological Co., Grand Island, New York.

Rates of Virus Accumulation in Cell Culture Medium

Purpose of experiment

Independent of the effects of overlay composition, the rate of increase in plaque size is limited by the rate at which newly-synthesized virus accumulates in the cell culture medium. This additional factor which could possibly affect the plaque-size properties of the Records variants was investigated by following the procedures outline by Dulbecco and Vogt (16) for one-step growth curve experiments with WEE virus on chicken embryo cells.

Experimental procedure

Cultures grown from the same lot of cells were placed in three groups. Each group was inoculated with one of the three Records variant populations. The multiplicity of infection was estimated to be between 5.0 and 10.0, so that nearly all susceptible cells in each culture would be initially infected. After 30 minutes for virus adsorption, the cultures were washed twice. Five ml. of fluid maintenance medium were then added to each culture, and zero-time samples of the medium were immediately taken from two cultures in each group. The remaining cultures were returned to the incubator. During the subsequent 18-hour incubation period, additional duplicate samples of maintenance

medium were harvested from two cultures in each group at two-hour intervals. All samples were immediately frozen at -40° C. until they could be titrated for virus content.

Multiplicity of infection

To determine the actual multiplicity of infections, the three test inocula were each further diluted for titrations by the plaque technique on additional cultures from the same lot. From these titrations, the titer of each Records variant used in the experiment was determined to be as follows: LP = 1.60×10^9, MP = 3.84×10^9, and SP = 9.55×10^8 plaque-forming-units (PFU) per ml.

The cells from 12 uninfected cultures were used for separate direct cell counts, and the average of the 12 counts became the estimated over-all average number of cells per culture. After growth medium was removed, 1.0 ml. of a solution of Versene[a] (ethylene diamine tetra acetic acid tetra sodium dihydrate, 1:2,000) and trypsin[b] (0.2 percent) in phosphate buffered saline was added to each plate. Each suspension of released cells was immediately diluted 1:10 in standard diluent. After

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[a]Nutritional Biochemicals Corp., Cleveland 28, Ohio.

[b]Difco Laboratories Inc., Detroit 1, Michigan .

mixing, a sample of each diluted cell suspension was counted in a hemocytometer. The counts varied from 3.7×10^6 to 4.9×10^6 cells, giving an average of 4.2×10^6 cells per culture.

The multiplicity of infection with each variant was calculated by dividing the number of PFU in each inoculum by the average number of cells per culture. These values were as follows: LP = 1.90, MP = 4.58, and SP = 11.38.

Determination of results

The virus titer of each maintenance medium sample was subsequently determined by the standard plaque assay procedure. Therefore the plaque-size properties of the virus which accumulated could also be determined.

Virulence Comparisons

Virus preparations

Plaque-cloned populations of each Records variant were propagated in CE cell cultures. When cytopathic effects were nearly complete, the viruses were harvested by removal of the fluid maintenance medium. Stock pools of each variant were dispensed in 2.5 ml. aliquots and frozen at -40° C. until titrations in several hosts could be performed.

Plaque titrations

Aliquots from each pool were titrated by the standard plaque assay. Both the titers (expressed as PFU per ml.) and the plaque-diameter distributions were compared.

Virulence for chicken embryos

Each Records variant was titrated, using five embryonated hens' eggs[a] per virus dilution. The procedure for intra-allantoic inoculation of ten to 11-day-old chicken embryos has previously been described (3). The relative virulence of each variant for the embryos was determined by comparing their infective titers (LD_{50} per ml.). Fifty percent endpoints were estimated, as in all subsequent titrations, by the method of Reed and Muench (49).

Virulence for baby chicks

Each Records variant was titrated in baby chicks by subcutaneous inoculation of 0.1 ml. of each virus dilution. Groups of five half-day-old chicks[b] injected with each dilution were separately housed in hatchery shipping cartons placed within modified Horsfall-Bower stainless steel isolation units. The inoculated chicks

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[a,b]Embryonated eggs and baby chicks were provided by Dr. M. S. Hofstad, Veterinary Medical Research Institute, Iowa State University, Ames, Iowa.

were fed, watered and observed at 12-hour intervals, and dead chicks were removed. The cardboard shipping cartons and their contents were incinerated, and the units were disinfected after each successive trial. The infective titer (LD_{50} per ml.) of each Records variant was determined. Samples of virus reisolated from the brains of moribund chicks after infection with the impure SP inoculum were later assayed by the standard plaque technique.

Virulence for mice

Weanling mice, obtained from Simonsen Laboratories, Inc., White Bear Lake, Minnesota, were separately caged in groups of five mice each. The mice inoculated with the Records variants by the intraperitoneal route received 0.1 ml. doses of virus. Those injected intracranially were each given 0.03 ml. The mice were observed daily until no further deaths attributable to the virus occurred. The titer (LD_{50} per ml.) of each variant by each route on inoculation was calculated.

Comparison of Antigenic Properties

Identification of the Records variants

It was necessary to identify each of the variants as WEE virus, and also necessary to prove that none of them was a contaminating virus accidentally obtained

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Comparison of Antigenic Properties

Identification of the Records variants

It was necessary to identify each of the variants as WEE virus, and also necessary to prove that none of them was a contaminating virus accidentally obtained

during the serial passages of the Records strain in guinea pig kidney cell cultures or in mice. For this purpose, anti-WEE, anti-EEE and anti-SLE typing sera were obtained from the Communicable Disease Center, U. S. Public Health Service, Atlanta, Georgia. Serum samples were also collected and pooled from two young roosters after each had been infected with a plaque-purified LP variant inoculum (1.0 ml., containing 2.0×10^6 PFU). The specificity of these four antisera for each of the Records variants was determined by a virus neutralization test, performed by a procedure which will later be described.

Preparation of inactivated Records variant antigens

Plaque-cloned populations of each Records variant were propagated in CE cell cultures, using serum-free maintenance medium. The medium was collected from each set of infected cultures when cytopathic effects were nearly complete, and also at this time from a set of uninfected control cultures. Cellular debris was sedimented from each fluid pool by centrifugation at 1,000 rpm. for 20 minutes. Several 2.5 ml. aliquots of each of the resulting supernatant fluids were immediately frozen. The remaining supernatant fluids were used for antigen preparation. To each, 10 percent formalin was added in amounts sufficient to produce a final 0.2 percent

concentration. The resulting LP, MP, SP and control antigens were stored at 5° C. for two weeks prior to their initial use.

Guinea pig immunization

Each of the four antigens was injected into three young adult guinea pigs, according to the time schedule shown in Table 6.

Table 6. Immunization and bleeding of guinea pigs

Days elapsed	Antigen[a] dosage	Route of inoculation	Blood[b] collected
0	0.2	subcutaneous	none
7	1.0	subcutaneous	none
14	2.0	intraperitoneal	5.0
35	none	none	10.0

[a]Milliliters, to each guinea pig.

[b]Milliliters from each, by cardiac puncture.

Guinea pig antisera

After each bleeding, the sterile serum from each group of three guinea pigs was pooled, and identified as LP, MP or SP antiserum or control serum. Each pool was then frozen. Serum neutralization tests were performed with the 14-day sera only. Samples from both 14-day and 35 day bleedings were titrated by hemagglutination-inhibition tests.

Neutralization tests

So that plaques could be readily counted after the performance of serum neutralization tests, it was essential that each control culture (from the set inoculated with a 1:1 mixture of control serum and virus) should receive approximately 50 PFU of virus. After samples of each Records variant were titrated by the standard plaque assay, similar samples were sufficiently diluted so that each contained an estimated 200 PFU per ml. Thus, after mixing of a 1.0 ml. aliquot with an equal volume of control serum, the desired number of virus particles was contained in 0.5 ml. of inoculum.

Before testing, each serum sample was heated to inactivate complement, and then initially diluted 1:10. Serial master dilutions in five-fold steps through 1:1250 were then made, and 1.0 ml. aliquots of each dilution were dispensed into separate tubes before

addition of virus.

The serum and virus mixtures (2.0 ml. total volume) were incubated for 30 minutes at room temperature. At the end of this time, unneutralized virus was titrated by three replicate platings (0.5 ml. each) from each test mixture on CE cell cultures. The standard plaque assay procedure was used.

All neutralization tests had thus been performed by the beta procedure, using constant amounts of each Records variant and testing against serial five-fold antiserum dilutions. The resulting antiserum titers were expressed as the reciprocal of that dilution which, in comparison to the average result with all control serum dilutions, caused a 50 percent reduction in the number of counted plaques.

Hemagglutination tests

Hemagglutination (HA) tests were performed by following essentially the same procedures as those described by Clarke and Casals (8). The borate-buffered saline (BBS), the phosphate-buffered saline (PBS) and the acid-citrate-dextrose (ACD) solutions used in the tests were prepared according to their suggested formulas. Only the points of departure from their widely-accepted standard procedures will be described.

Plaque-cloned populations of each Records variant

were propagated in CE cell cultures, using serum-free maintenance medium. Cultures were washed twice with serum-free diluent prior to inoculation with virus. Each virus was harvested when cytopathic effects in the cultures were nearly complete. Cell debris was sedimented from the medium harvested from infected cultures by centrifugation at 1,000 rpm. for 20 minutes. Samples of each supernatant fluid were frozen for later plaque titrations. Additional samples were diluted 1:10 in BBS at pH 9.0, and stored at 5° C. for later use as HA test antigens. Further 2-fold dilutions of each antigen in BBS were made prior to each test.

Erythrocytes used in the HA test were obtained by bleeding day-old chicks into physiological saline solution containing 0.4 percent sodium citrate. The cells were immediately sedimented and resuspended in ACD solution for storage at 5° C. Just before use, they were washed three additional times in ACD solution. Final suspension was in a PBS solution with phosphate buffer concentrations appropriately chosen so as to establish a final pH of 6.0 after mixing of cells and virus. The concentration of red blood cells in the resulting suspension was 0.25 percent, based on packed cell to total fluid volume.

The test was performed by mixing 0.5 ml. of each

antigen dilution with an equal volume of suspended cells and incubating for two hours at room temperature. Tests were read at this time by observing the tube containing the highest virus dilution in which complete hemagglutination had occurred. One HA unit was designated as the smallest amount of virus antigen required to completely agglutinate the standard amount of chick erythrocytes under the conditions described.

Hemagglutination-inhibition test

Samples of each pooled guinea pig serum were prepared for use in the hemagglutination-inhibition (HI) test by following the adsorption procedures described by Clarke and Casals (8). Non-specific inhibitors of hemagglutination were adsorbed with kaolin[a]. Naturally-occurring agglutinins for chicken erythrocytes were then adsorbed by treatment with packed, washed red blood cells from day-old chicks. Starting with an initial 1:10 dilution, serial 2-fold dilutions of each serum were made. Aliquots (0.25 ml.) of each were then dispensed into separate tubes for addition of antigen.

To each serum dilution, 0.25 ml. of virus antigen, sufficiently diluted in BBS so as to contain 4 HA units, was added. Serum and virus mixtures were incubated

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[a]Jay E. Cook, Importer, Cockeysville, Maryland.

overnight at 5° C. prior to the addition of 0.5 ml. of a 0.25 percent suspension of chick erythrocytes. Subsequent incubation and reading of the tests was by the procedure described for hemagglutination.

Antiserum titers were expressed as the reciprocal of the highest dilution causing complete inhibition of hemagglutination, under the conditions described.

RESULTS

Plaque Diameter Comparisons[a]

The parent strain

A sample of the Records strain of WEE virus, which had been kept frozen in chicken embryo allantoic fluid for 15 years, was titrated by the standard plaque assay procedure. Its titer, after storage for that length of time, was 2.2×10^8 PFU per ml. The plaques ranged in size from 2.0 to 5.0 mm., and the mean plaque diameter was 3.9 mm. This then, was the characteristic plaque size of the Records strain as it existed in 1951, before any selective cloning experiments were performed.

Results of selective cloning

The typical appearance of plaques produced by each of the Records variants in CE cell cultures under standard assay conditions is shown in Figure 1. Each type of plaque may be clearly distinguished from the others on the basis of size.

Also after titration by the standard assay procedure, the range in size and the mean plaque diameter of each of the Records variants can be graphed as in Figure 2. Differences between the mean plaque diameters were tested for significance by the T-test following the method

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[a]Complete data may be found in the Appendix.

Figure 1. Typical appearance of plaques produced by each of the Records variants under standard plaque assay conditions

LP variant

MP variant

SP variant

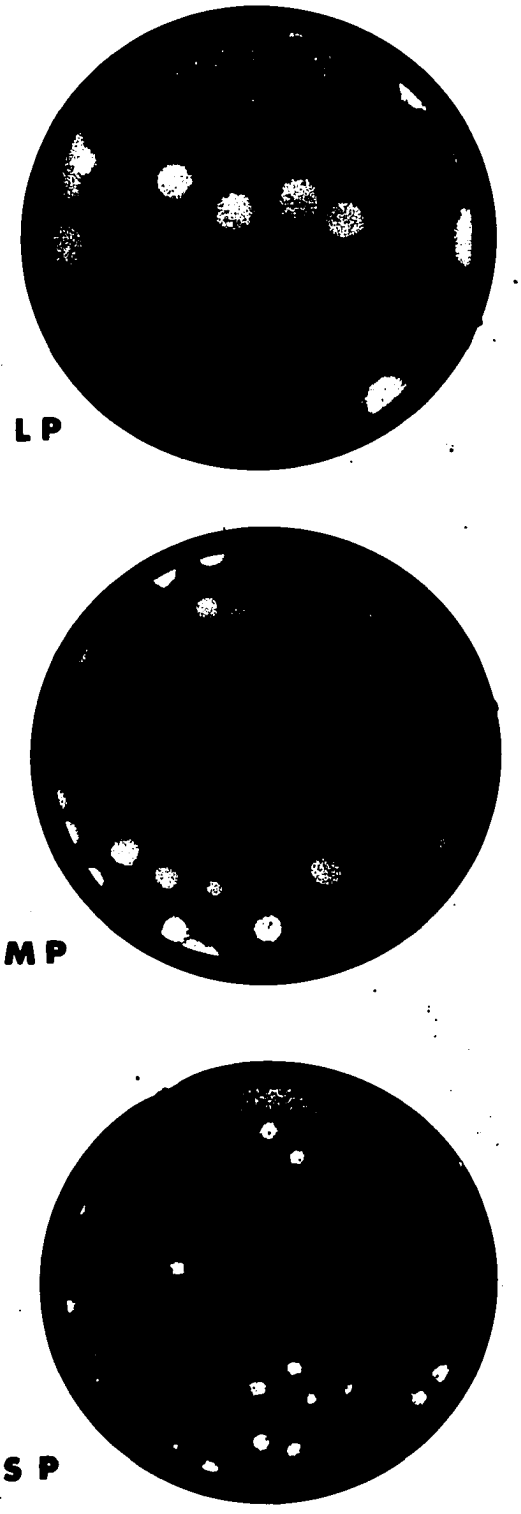
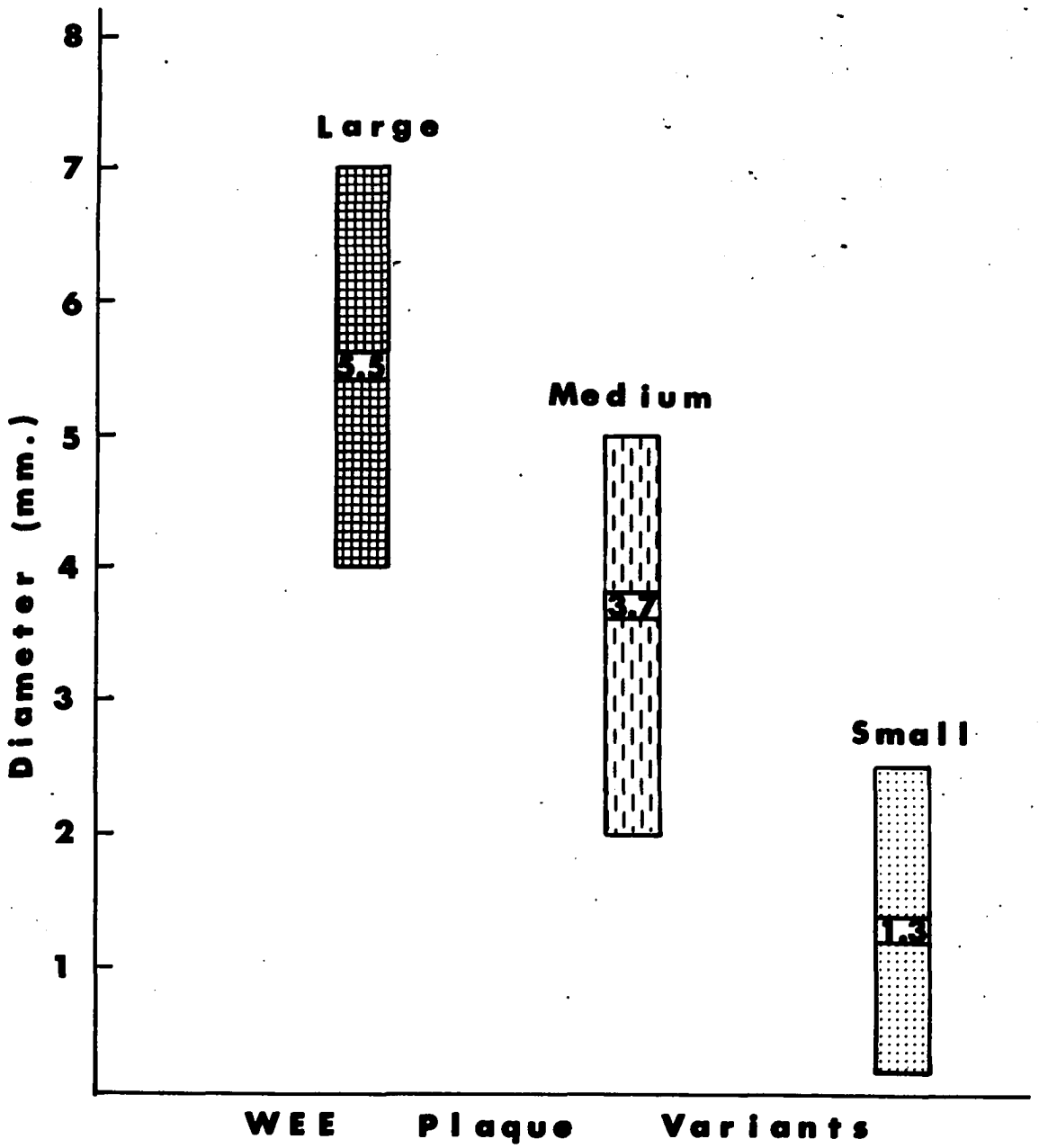

LP
MP
SP

Figure 2. Plaque diameter range and mean of each Records variant under standard conditions


8
7
6
5
4
3
2
1
Diameter (mm.)
Large
5.5
Medium
3.7
Small
1.3
WEE Plaque Variants

of Snedecor (53), and were found to be significant at the 99 percent level of probability.

The MP variant produces plaques very closely approximating the diameter of those produced by the parent virus. As it was used in the current study, the MP variant was apparently a plaque-purified population after having undergone four serial clonings in CE cell cultures.

The LP variant was detected for the first time after a sample of the Records strain had been serially passed in guinea pig kidney cell cultures. As previously reported (3), there was evidence that it existed as an incompletely purified population after three serial selective clonings. It was purified however, as a result of seven additional serial clonings, and it was an apparently pure LP population which was investigated in the project being reported.

The SP variant first appeared in a sample of the Records strain which had undergone eight serial intracranial passages in weanling mice. It has thus far been impossible to plaque purify SP variant populations by selective cloning in CE cell cultures. The presence of a MP impurity at a level of one to two percent of the total plaques counted was a consistent finding when selectively-cloned SP populations were titrated.

Properties of seven recent WEE virus isolates

The range in size and the mean plaque diameters of seven recent WEE virus isolates, and those of the three Records variants are compared in Figure 3. The field strains tested were isolated in several states and from several different host species. It can be seen that the mean plaque diameter of each recent isolate was not far different from that of the LP variant. However there was usually a broader size range, and nearly every isolate produced some plaques resembling the MP type. In no case, were small plaques observed.

Several of the field strains produced plaques which appeared to fall into two distinct size groupings. This result is indicated in Figure 3 by the bar graphs of plaque-size distribution which contain central unshaded segments. Additional evidence for this apparent plaque-size heterogeneity was obtained as a result of a second CE cell culture passage, since the progeny virus harvested from either large or medium-type plaques again produced plaques ranging widely in size and again consisting mostly of the LP type. The rather limited trials did not result in the selective cloning of a MP variant from any of the field strains.

Figure 3. Comparison of the plaque diameters of seven recent isolates with those of the Records variants

Letters designate the strains and sources of isolation as follows:

L. Records strain, LP variant

M. Records strain, MP variant

S. Records strain, SP variant

A. 65V-241, whole blood, Passer domesticus, Texas

B. 65V-796, whole blood, Passer domesticus, Texas

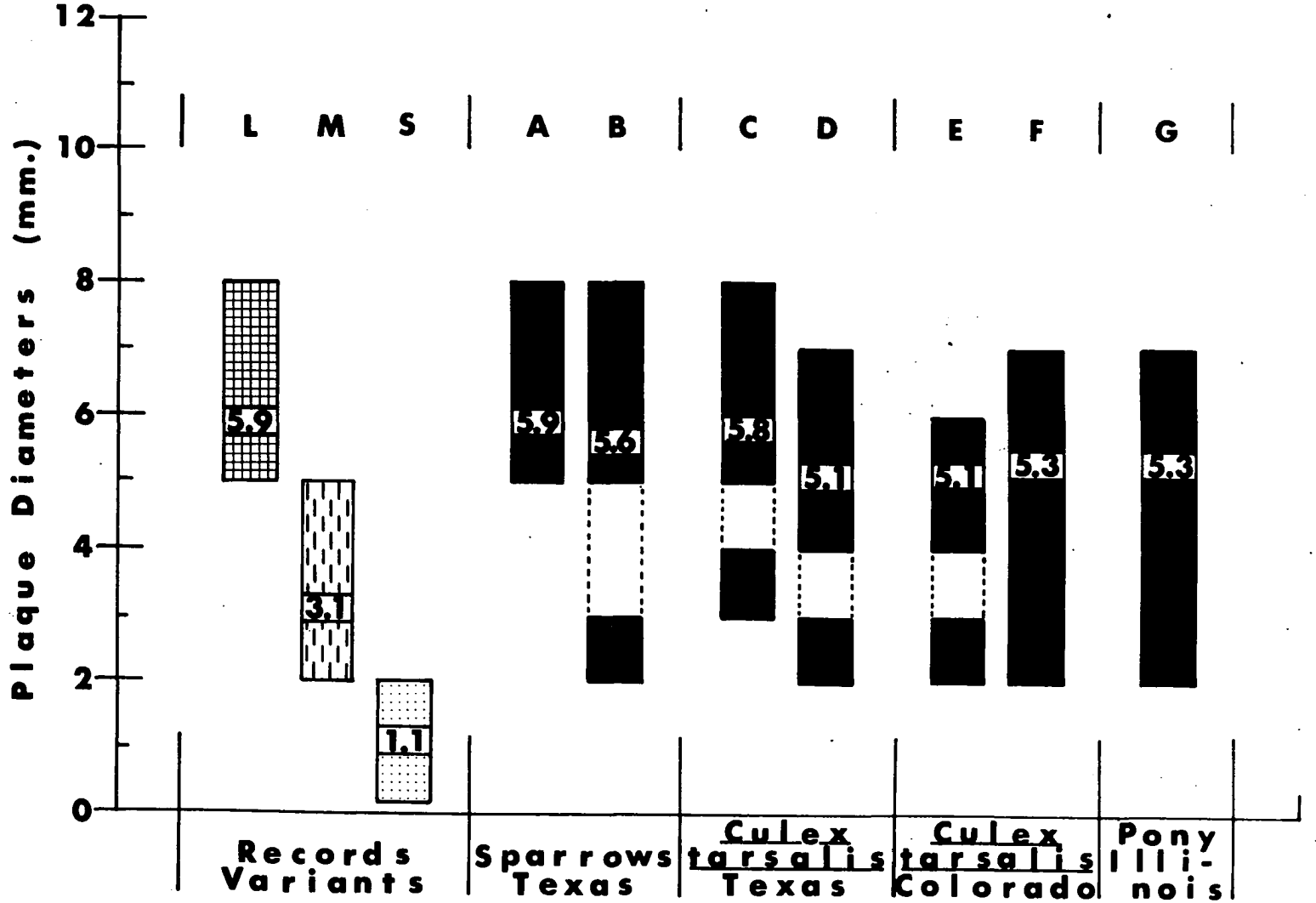
C. 65V-657, Culex tarsalis pool, Texas

D. 65V-692, Culex tarsalis pool, Texas

E. 65V-633, Culex tarsalis pool, Colorado

F. 65V-629, Culex tarsalis pool, Colorado

G. E 2329, pony brain, Illinois


Plaque Diameters (mm.)
12
10
8
6
4
2
0
L
M
S
A
B
C
D
E
F
G
5.9
3.1
1.1
5.9
5.6
5.8
5.1
5.1
5.3
5.3
Records Variants
Sparrows Texas
Culex tarsalis Texas
Culex tarsalis Colorado
Pony Illi-nois

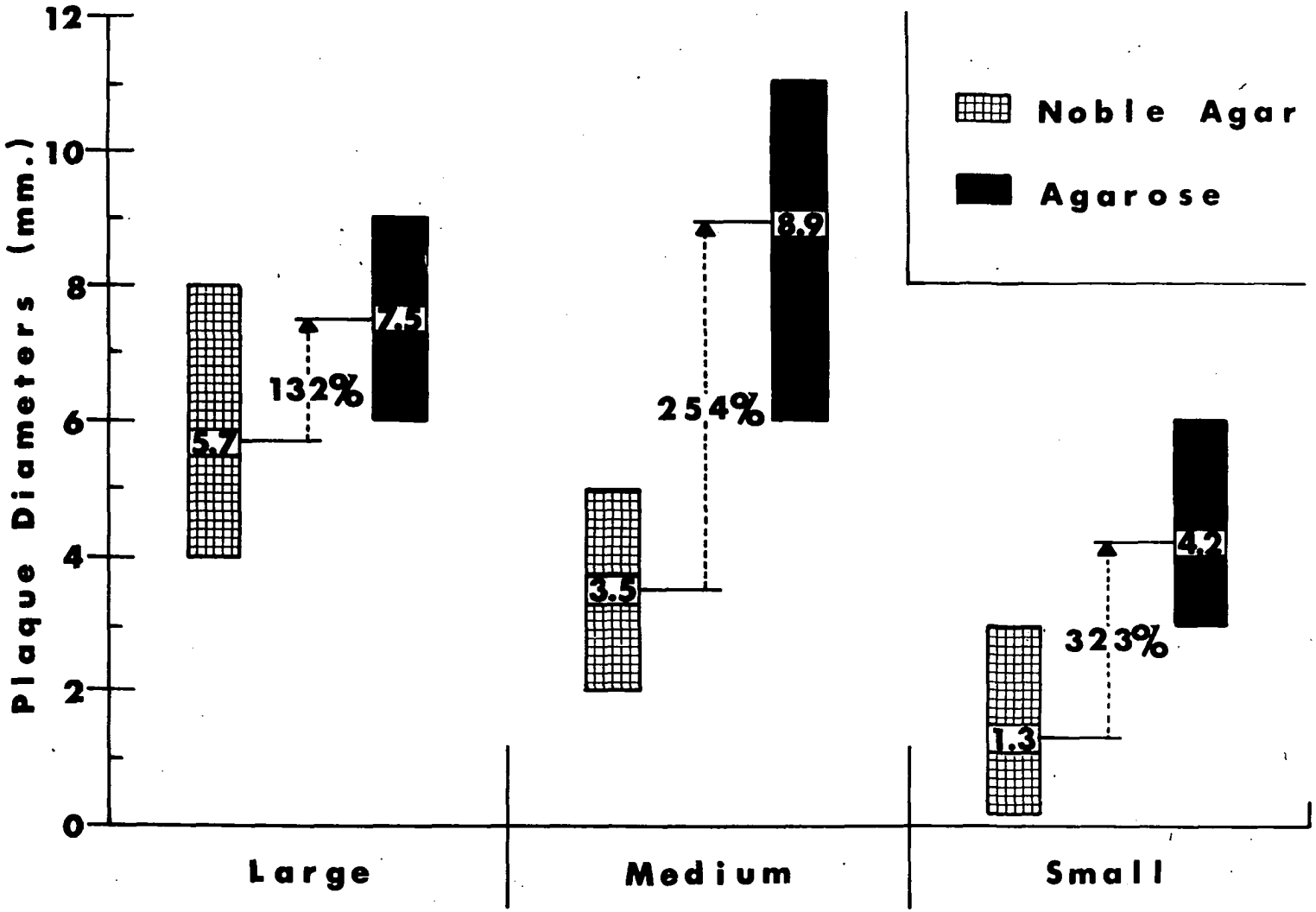
Effect of agarose overlays

The typical mean plaque diameter of each of the Records variants was increased when these viruses were titrated under overlays containing agarose in place of Noble agar. The relative magnitude of this increase is graphed in Figure 4. The percent increase in mean plaque diameter is also indicated, but is not graphically represented.

The LP variant, known to be relatively insusceptible to the effects of the agar inhibitor, therefore exhibited the smallest plaque-size increase when inhibition was removed. Under overlays with reduced sulfate ion content, the MP variant produced plaques with an average diameter that surpassed even the LP type. The percent increase in diameter was greatest for the SP type, but the actual mean plaque diameter attained fell far short of this value for the other variants. As a result, the difference in size between small and medium plaques became even more distinctly observable under agarose overlays. Some other limiting factor, in addition to sulfated-polysaccharide inhibition apparently was responsible for restricting the development of plaques produced by the SP variant.

Differences between the mean plaque diameters of each variant under agarose overlays were also found to be significant at the 99 percent level of probability,

Figure 4. Relative increase in plaque diameters resulting from the substitution of agarose for Noble agar in the overlay medium


Plaque Diameters (mm.)
12
10
8
6
4
2
0
Noble Agar
Agarose
5.7
7.5
132%
3.5
8.9
254%
1.3
4.2
323%
Large
Medium
Small

54a

when tested by the method of Snedecor (53).

Effect of inhibitors added to agarose overlays

Various preparations containing sulfated polysaccharides were added to agarose overlay medium. Titration of each Records variant under each type of overlay yielded the results shown in Figure 5. The vertical arrows indicate the amount of change in the mean plaque diameter that can be attributed to each added preparation.

The characteristic plaque size of each variant under agarose control overlays (A) was initially compared. The addition of Noble agar extract (B) caused a reduction in the size of plaques produced by the MP and SP variants without noticeably decreasing the mean plaque diameter of the LP type. In the presence of Bacto agar extract (C), small and medium plaques were further reduced in size. A reduction in the average plaque diameter of the LP variant did occur, but to a lesser degree. A significant decrease in the average plaque size of all the variants was observed in the presence of the overlay medium containing 100 micrograms of sodium dextran sulfate per ml. (D). Under this type of overlay, differences in plaque diameter between the three Records variants were virtually non-existent.

Figure 5. Effect on plaque diameters of inhibitory substances added to agarose overlays

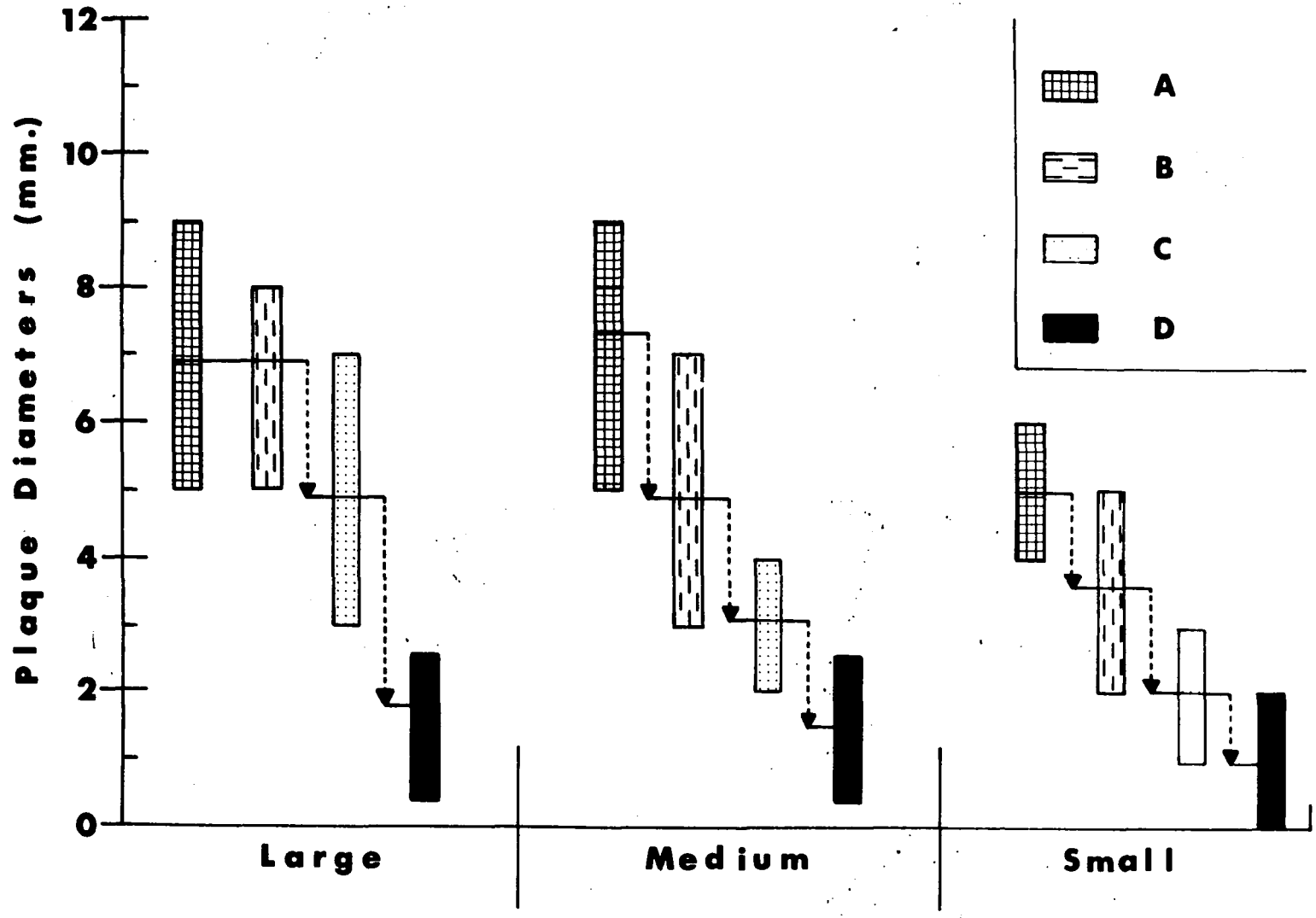
Letters indicate the following preparations:

A. Agarose extract

B. Noble agar extract

C. Bacto agar extract

D. Sodium dextran sulfate


Plaque Diameters (mm.)
12
10
8
6
4
2
0
A
B
C
D
Large
Medium
Small

Effects of cysteine and lactalbumin hydrolysate added to skim milk overlays

Figure 6 is a graph of the plaque diameter range and mean of each variant determined by titrations under skim milk overlays with various modifications. The increase in mean plaque diameter which resulted from each modification of the basal overlay medium is illustrated by a vertical arrow.

The bar graphs in group A indicate the plaque-size properties of each variant under a basal skim milk overlay containing Noble agar and no additives. The smaller-than-usual plaques (smaller than those produced by the three variants under standard assay conditions) were apparently not the result of deficient nutritive properties of the skim milk maintenance medium. This fact was established by the observance of the significantly larger plaques (D) which were produced in the same skim milk medium changed only by substituting agarose for Noble agar.

The addition of cysteine to skim milk and Noble agar overlays (B) caused an increase in the average diameter of each type of plaque. It also was observed (as may be seen from a comparison of the mean plaque diameters graphed in groups A and B of Figure 6) that the relative increase was greatest for the LP variant.

Figure 6. Relative increase in plaque diameters resulting from changes in the composition of skim milk overlay medium

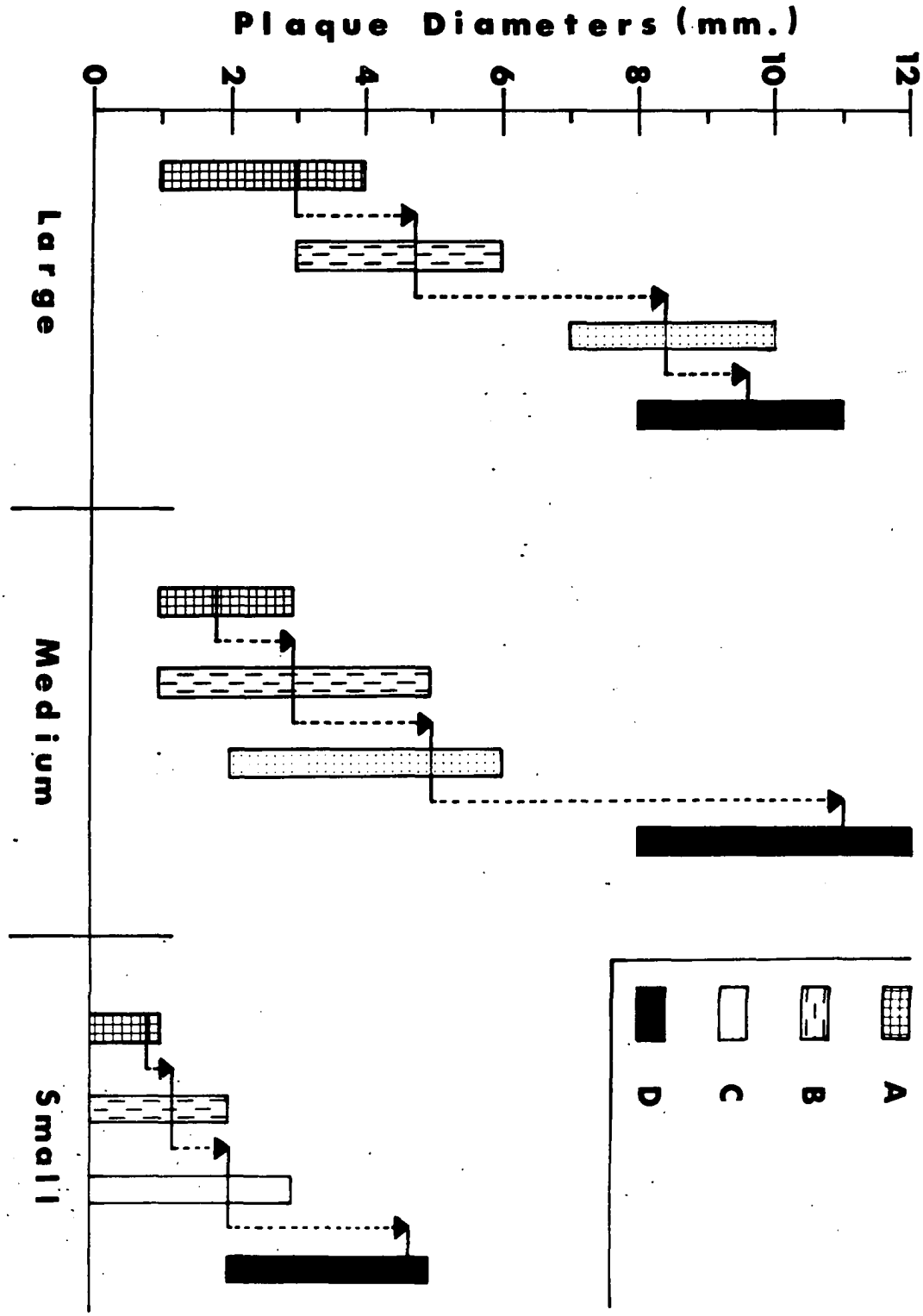
Letters indicate media of the following compositions:

A. Skim milk with Noble agar

B. Skim milk with Noble agar plus cysteine

C. Skim milk with Noble agar plus lactalbumin hydrolysate

D. Skim milk with agarose


Plaque Diameters (mm.)
0
2
4
6
8
10
12
Large
Medium
Small
D
C
B
A

Lactalbumin hydrolysate, when added to skim milk and Noble agar overlay medium (C), was responsible for a further increase in plaque size. The range in plaque size for each variant under this type of overlay was nearly identical to that typically observed under the standard plaque assay conditions. The LP variant, in the skim milk, lactalbumin hydrolysate and Noble agar medium, produced plaques with a mean diameter nearly equal to those which it produced under agarose overlays. It can be noted in Figure 6 (C and D) that this was not the case with the MP and SP variants. Only a relatively small increase in the medium and small plaque types resulted from the addition of lactalbumin hydrolysate.

Effects of bovine serum

The effect of bovine serum in the overlay medium has been found to vary, depending upon the variant titrated and upon whether agarose or Noble agar overlays were used. These different effects on plaque development are shown in Figure 7. As in previous graphs, the arrows are used to indicate the amount of increase or decrease in the mean plaque diameter of each variant.

As indicated in Figure 7, there was little, if any, change in the mean plaque diameter of the SP variant when serum was added to either Noble agar or agarose overlay medium. In either medium, 5 percent bovine serum

Figure 7. Relative changes in plaque diameters resulting from the addition of bovine serum to Noble agar or agarose overlays

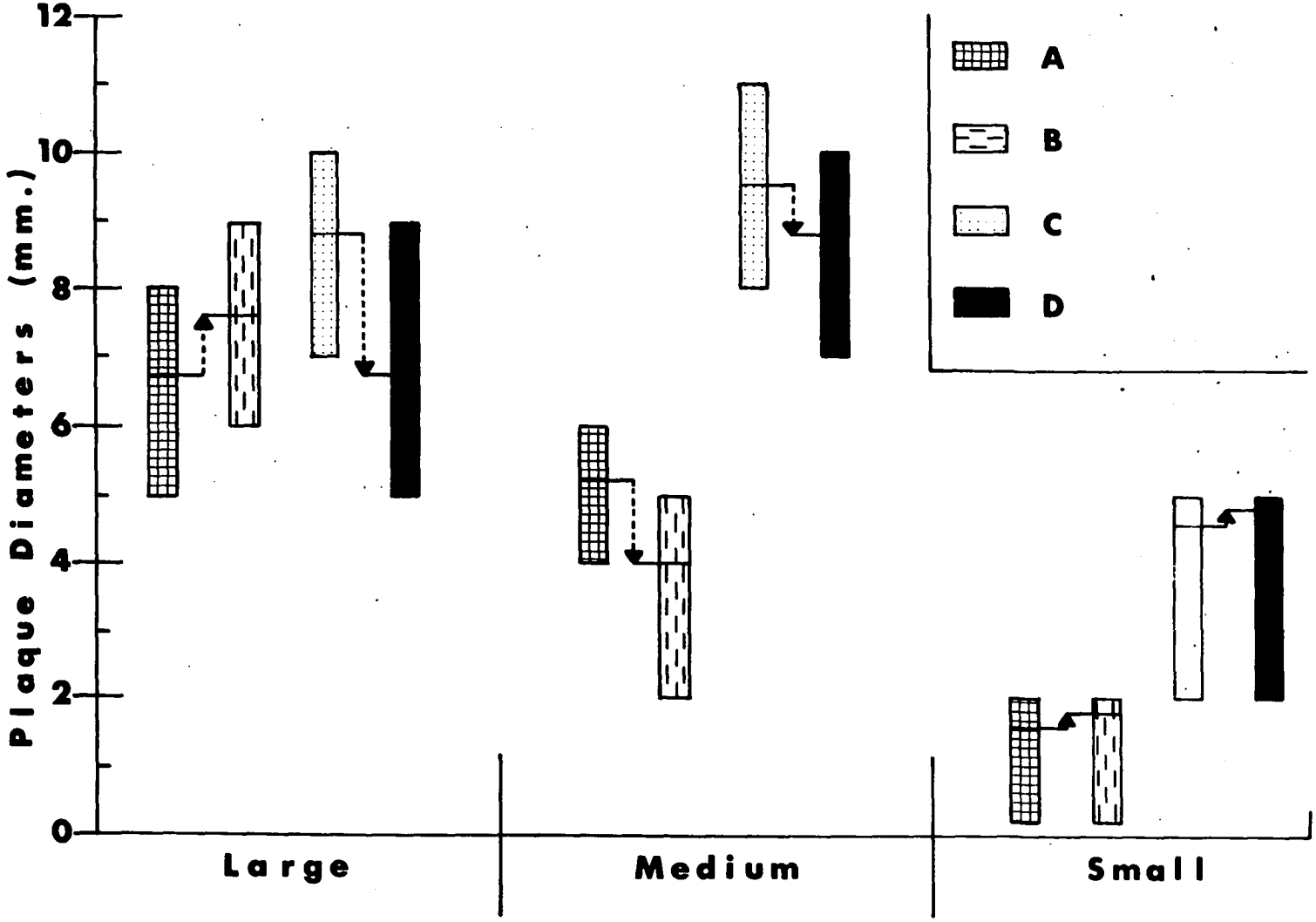
Letters indicate the following:

A. Noble agar overlay without serum

B. Noble agar overlay with added bovine serum

C. Agarose overlay without serum

D. Agarose overlay with added bovine serum


12
10
8
6
4
2
0
Plaque Diameters (mm.)
A
B
C
D
Large
Medium
Small

slightly inhibited the development of medium type plaques. The results with the LP variant presented still another variation. In this case, development of large plaques was enhanced by serum under Noble agar overlays, but was inhibited by serum under agarose. A possible reason for these different results will be discussed.

Effect of CE cell culture passage on the SP variant

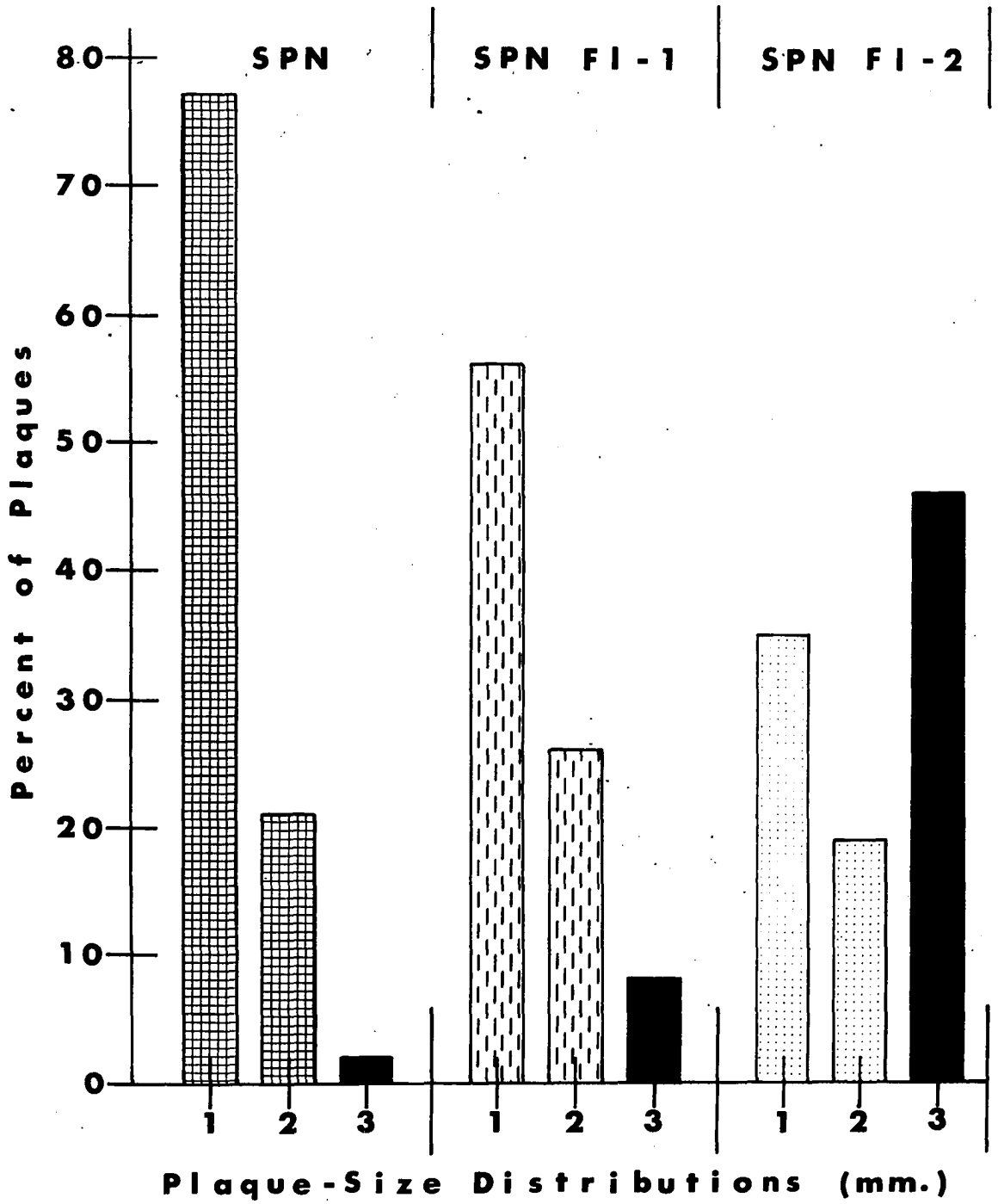
While the plaque-cloned populations of either the LP or MP variant were not observed to change significantly during as many as four serial passages in CE cell cultures, this has not been true of the SP variant populations thus far obtained. In Figure 8, an example of typical results obtained with serial cell culture passage of a SP population is shown.

The original population (SPN), after 3 serial clonings in CE cultures, produced primarily small plaques, 77 percent of which were 1.0 mm. or smaller in diameter. Only 2 percent (shown as the first darkly shaded bar in Figure 8) were over 2 mm. in diameter, and thus fell into the MP range. After two serial passages in CE cell cultures using fluid maintenance medium (SPN Fl-1 and SPN Fl-2), the resulting population was found to produce approximately 46 percent medium plaques (again demonstrated by the darker shading). The growth rate study which follows, was initiated as

Figure 8. Progressive increase in the proportion of medium plaques resulting from two serial cell culture passages of a SP population

Letters identify the passage levels as follows:

SPN.	Nearly pure SP population resulting from three serial clonings
SPN F1-1.	SPN population after 1 cell culture passage
SPN F1-2.	SPN F1-1 population after 1 additional cell culture passage


SPN
SPN FI-1
SPN FI-2
80
70
60
50
40
30
20
10
0
Percent of Plaques
1
2
3
1
2
3
1
2
3
Plaque-Size Distributions (mm.)

an attempt to determine a possible cause for this rapid increase in the proportion of the MP variant which accompanied cell culture passage.

Rates of Virus Accumulation in Cell Culture Medium

Total free virus

After infection of separate groups of CE cell cultures with the Records variants, samples of the fluid maintenance medium from each group of infected cultures were harvested at 2-hour intervals. The titer of accumulated virus in each sample (expressed as the Log_{10} PFU per ml.) was determined, and was plotted versus time in Figure 9. The virus growth curves, as drawn, indicate only the total virus titrated, and do not take into consideration the plaque types observed on the titration plates.

Populations of the LP and MP variant have been successfully plaque-purified as a result of selective cloning. The rates of accumulation of these two variants could therefore be studied in this experiment by using plaque-purified inocula, and the plaques observed on the cultures used to titrate accumulated virus were either 100 percent large or 100 percent medium type plaques, depending on the inoculum used. The results obtained

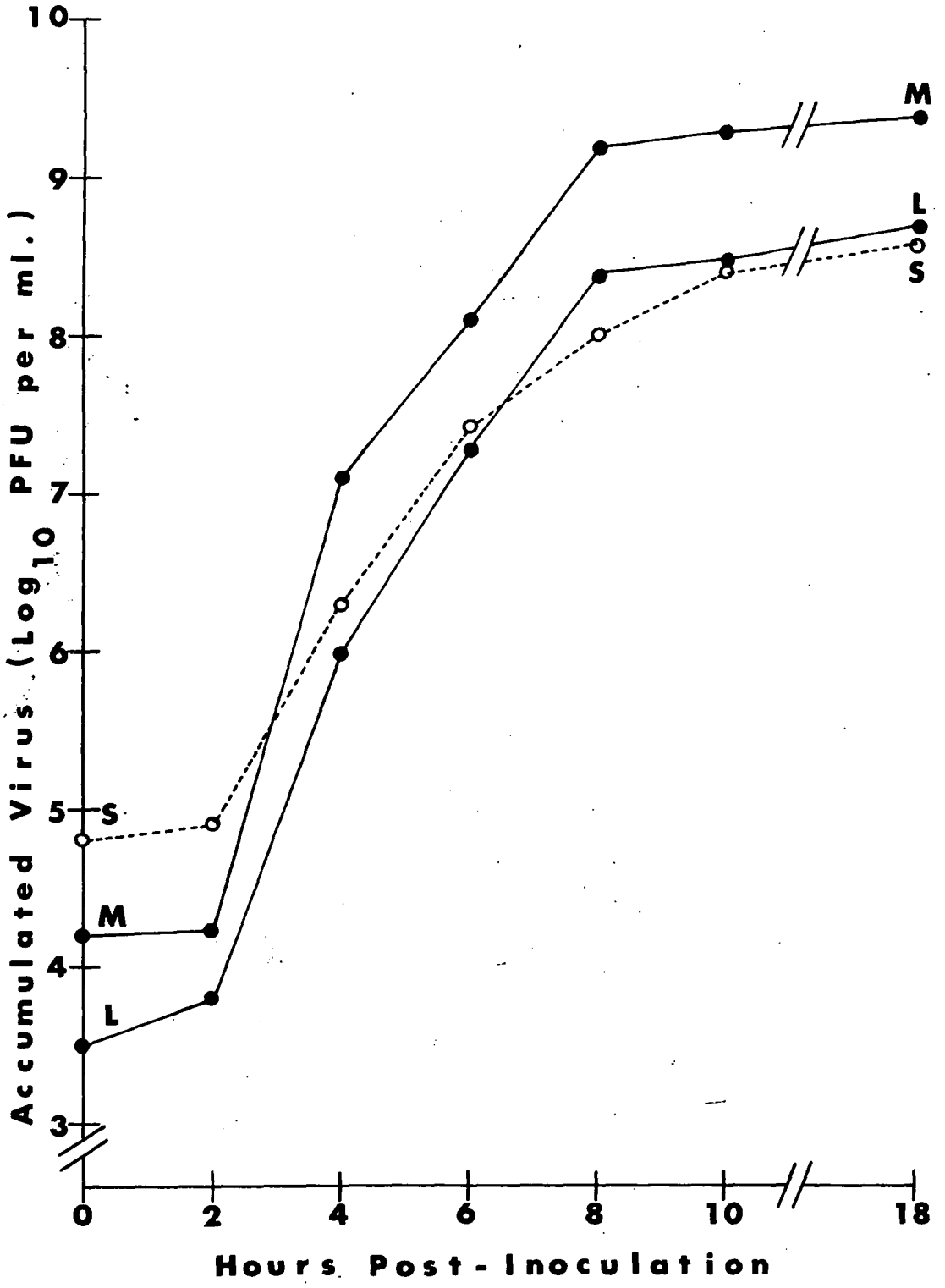
Figure 9. Titers of virus accumulated in the fluid maintenance medium of cell cultures at intervals following infection

Letters indicate the following:

L. Pure LP variant

M. Pure MP variant

S-M. SP variant, with 4.6 percent MP impurity in inoculum


10
9
8
7
6
5
4
3
Accumulated Virus (Log10 PFU per ml.)
M
L
S
S
M
L
0
2
4
6
8
10
18
Hours Post-Inoculation

with these two variants can therefore be directly compared and analyzed. The growth rate curves for the pure LP and pure MP variants are thus depicted as solid lines and closed points in Figure 9.

In contrast, it has not been possible to obtain pure populations of the SP variant. As a result, it should be noted that the SP variant preparation used as the inoculum in this experiment contained a MP variant impurity which was determined to approximate 4.6 percent of the total population. When many medium-sized plaques appeared on the cultures inoculated with this set of maintenance medium samples, it became obvious that this impurity in the SP inoculum was of some significance. Since curve S-M, as depicted in Figure 9, results only from considering total accumulated virus, it is not a valid indication of the true growth rate of the SP variant. Instead, it represents the combined total of medium and small plaques which resulted from a mixed infection. Since this result cannot validly and directly be compared with those obtained for the other two variants, curve S-M is drawn with open points connected by a broken line in order to distinguish it from the other curves, and to indicate that results from this set of titrations will be analyzed independently.

Rates of accumulation of the LP and MP variants

Again, in reference to Figure 9, it can be observed that the period of most-rapid increase in accumulated virus was from 2 to 8 hours post-infection with either the LP or MP variant. By linear regression analysis, best-fitting straight lines could be superimposed on this relatively linear portion of each curve, as has been done in Figure 10. The slope of each line was taken as the best numerical estimate of the maximum rate of accumulation of each variant. The slope of line M (MP variant) is 0.78 and that for line L (LP variant) is 0.75, when calculated by the method of Snedecor (53). There is therefore a good indication that the rate of accumulation of the MP variant only slightly exceeds that of its LP counterpart as a result of separate cell culture infections with plaque-purified preparations.

Plaques observed after mixed SP-MP cell culture infection

When the plaques obtained from the SP growth curve experiment were classified according to diameter, the MP type as a percent of the total counted plaques could be plotted, as shown in Figure 11. While the MP impurity amounted to only 4.6 percent of the inoculum, it represented 7.5 percent of the free virus recovered at 2 hours after infection. Somewhat surprisingly, this figure rose to 86.0 percent of the plaques counted from samples taken

Figure 10. Relative rates of accumulation of the LP and MP variants in the fluid medium of cell cultures following separate infections with plaque-purified inoculua

Solid line M-M represents MP Records variant

Broken line L-L represents LP Records variant

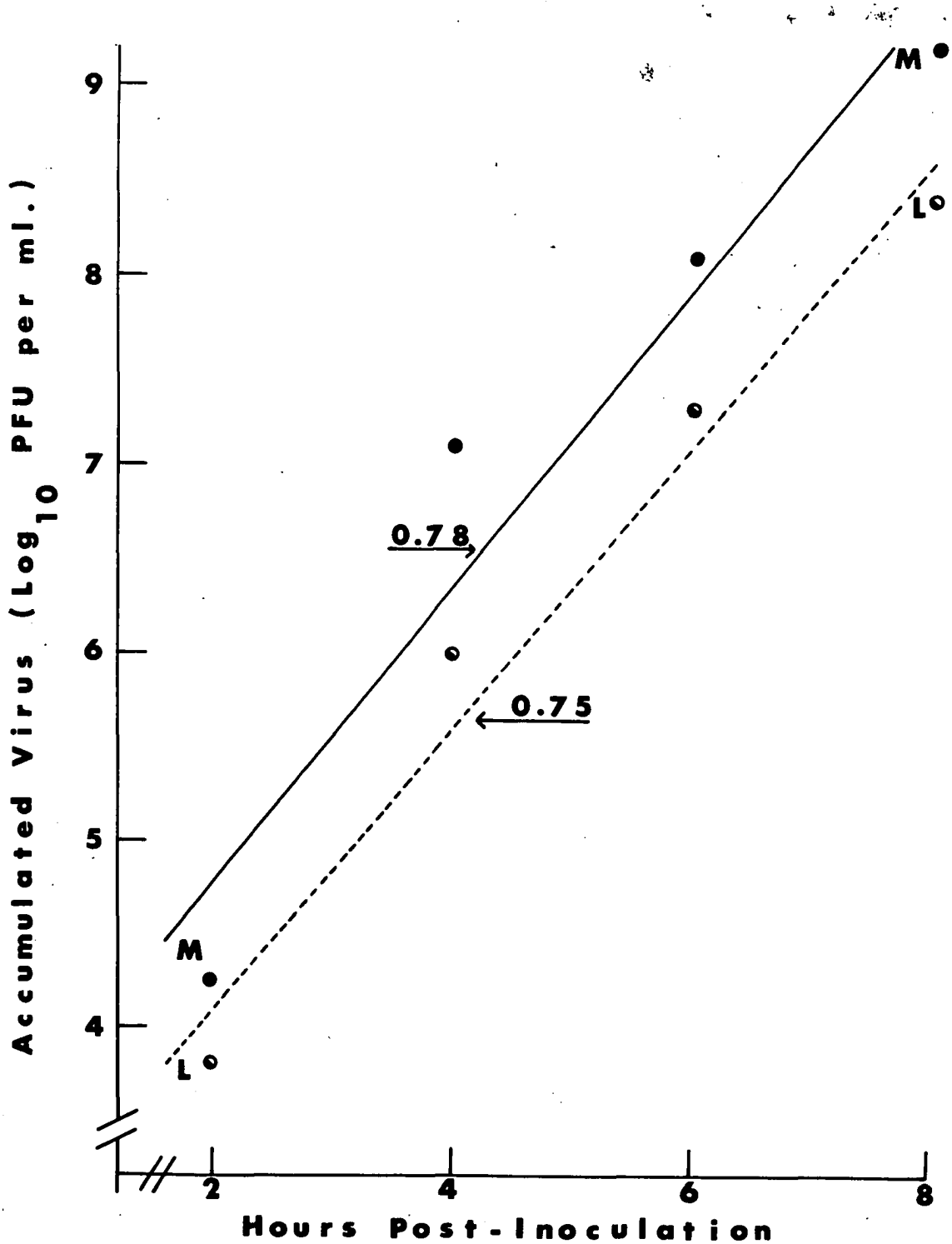
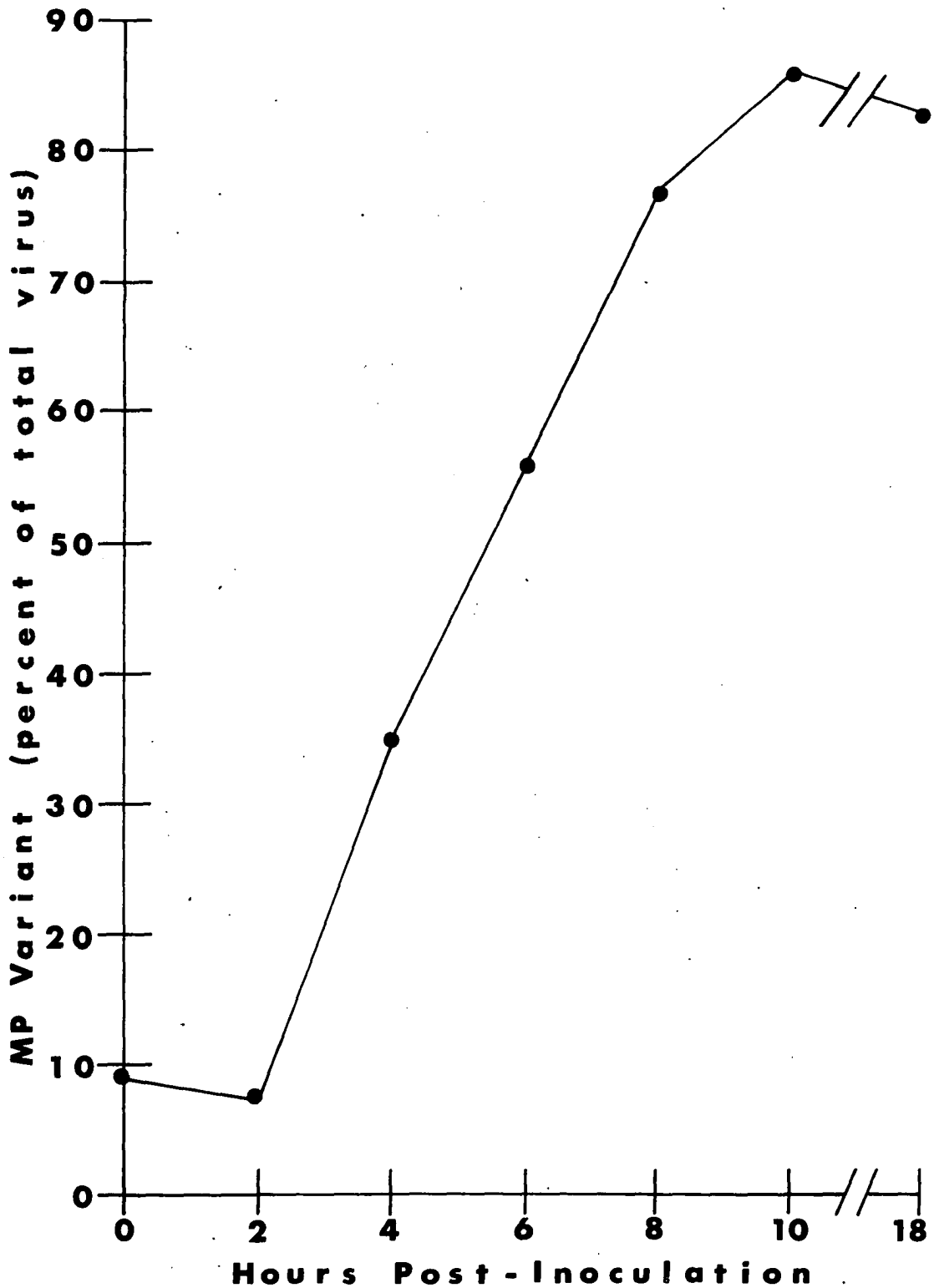

Accumulated Virus (Log10 PFU per ml.)
9
8
7
6
5
4
2
4
6
8
Hours Post-Inoculation
M
L°
0.78
0.75
M
L°

Figure 11. Medium plaque variant, as a percent of the total virus, accumulating in the fluid maintenance medium of cell cultures after infection with an impure SP inoculum


MP Variant (percent of total virus)
90
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
18
Hours Post-Inoculation

at 10 hours post-infection. This result suggested that the respective rates of accumulation of the MP and SP variants in this set of cultures should be more critically compared.

Rates of accumulation of MP and SP variants

Using the data already obtained, separate curves could be plotted for the MP and SP variants as each accumulated in cell culture maintenance medium following a mixed infection. They are illustrated in Figure 12. As expected, the two curves gave further indication of the difference in the rate of accumulation of each variant. As was observed in the case of infection with the plaque-purified variants, the period between 2 and 8 hours post-infection was the time of most-rapid increase in newly-replicated virus. Best-fitting straight lines could again be employed to estimate the rate differences between the variants. The slope of line M (MP variant) is 0.69, while that of line S (SP variant) is only 0.42, when both are calculated by the previously indicated method, and as they are drawn in Figure 13. It was apparently impossible to determine the actual rate of accumulation of the SP variant which might have resulted from cell culture infection with a plaque-purified preparation. It has at least been established however, that following a mixed SP-MP infection the MP variant accumulates in the

Figure 12. Titers of SP and MP variants accumulating in the fluid maintenance medium of cell cultures following infection with an impure SP inoculum

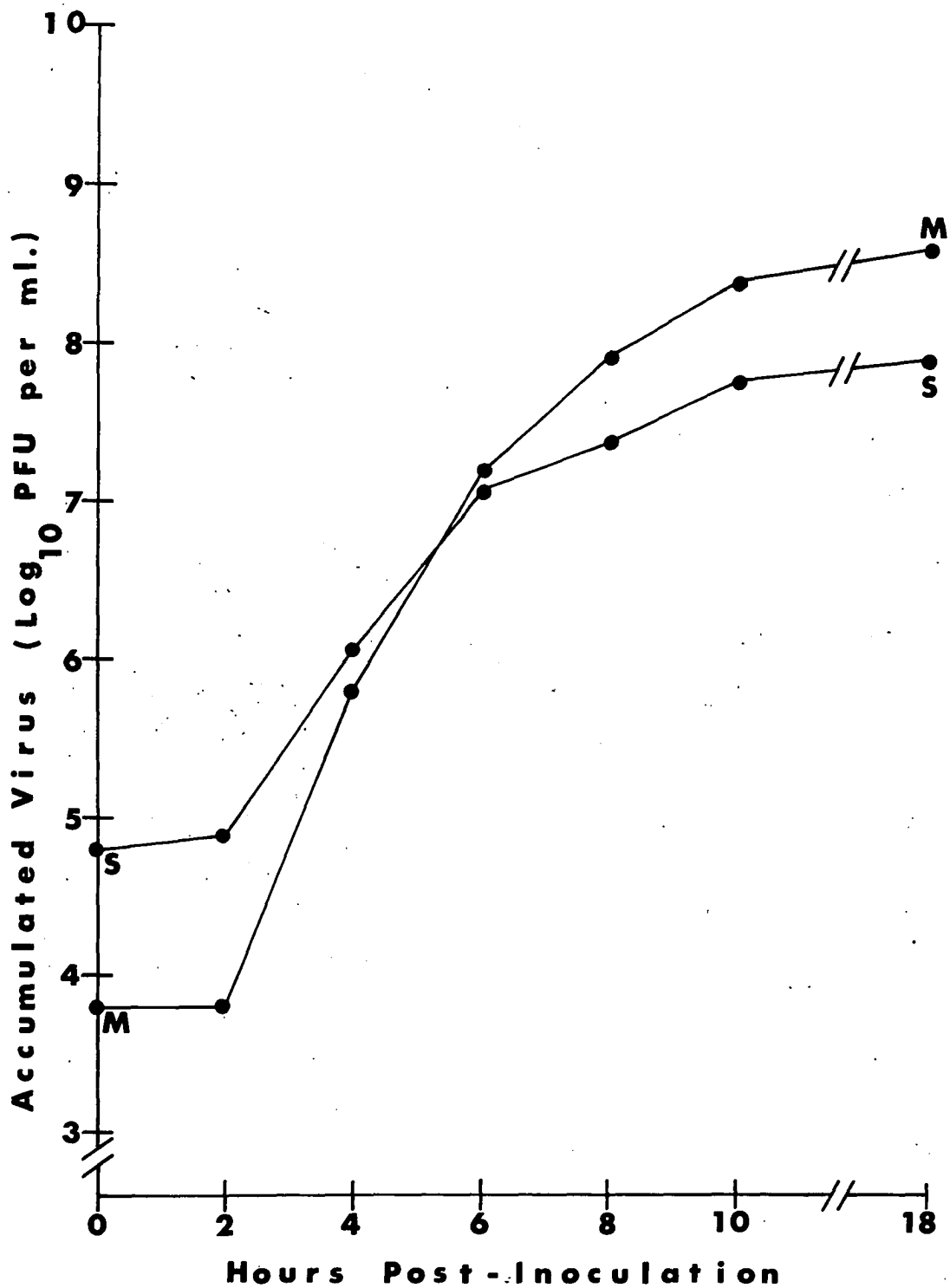
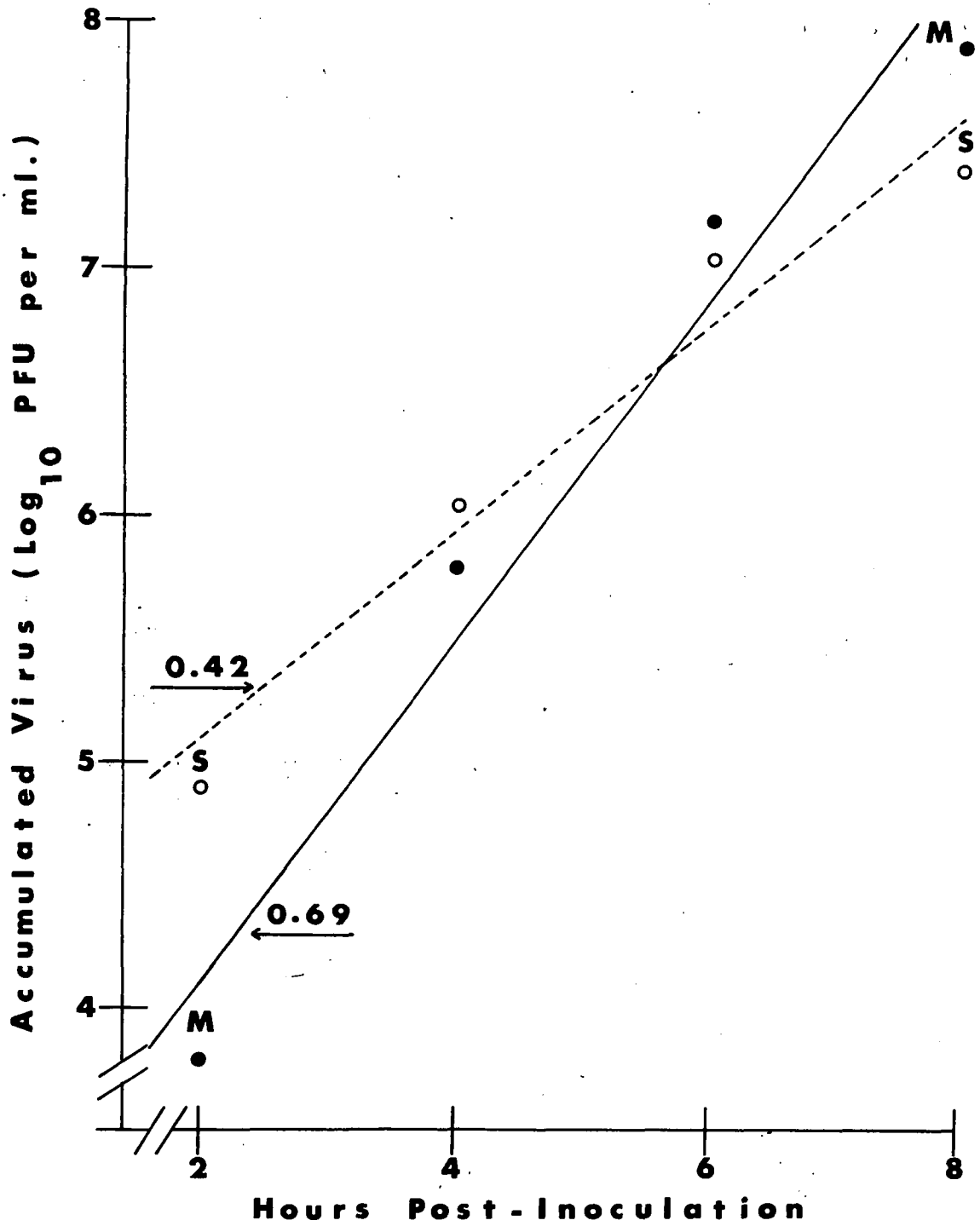

10
9
8
7
6
5
4
3
Accumulated Virus (Log10 PFU per ml.)
M
S
S
M
0
2
4
6
8
10
18
Hours Post-Inoculation

Figure 13. Relative rates of accumulation of the SP and MP variants in the fluid medium of cell cultures following infection with an impure SP inoculum


8
7
6
5
4
M
S
0.42
0.69
M
S
2
4
6
8
Accumulated Virus (Log10 PFU per ml.)
Hours Post-Inoculation

cell culture medium at a significantly higher rate.

Possible homologous interference

One additional rate comparison may be made when the results of the growth rate experiments are considered. It may be seen that the estimated rate of accumulation for the MP variant was initially 0.78, a value obtained from the results of cell culture infection with a plaque-purified MP inoculum. However, its estimated rate of accumulation after a mixed infection of cell cultures was calculated to be only 0.69. It is quite likely that the presence of the SP variant, and its interference with the replication of the MP variant could account for the rate differences observed.

Virulence Comparisons

Plaque titrations

The virus titer and the plaque-size properties of samples from each pool prepared for the virulence trials were determined by the standard plaque assay procedure before animal inoculations were begun. The titer of each Records variant was as follows: LP = 4.3×10^8, MP = 1.5×10^9 and SP = 3.4×10^8 PFU per ml. Since their titers were similar, approximately equal doses of each variant could be given to the test animals

by preparing identical dilutions of each Record variant. The LP and the MP populations produced 100 percent large and medium plaques respectively, since the selective cloning attempts had successfully resulted in the plaque-purification of these two variants. However, approximately 1.6 percent of the plaques observed after titration of the sample from the SP pool fell into the medium plaque size range, indicating once again that complete plaque-purification of the SP variant population had not been accomplished. Since this was about the highest degree of purity obtainable with a SP preparation after propagation in CE cell cultures, it was decided to proceed with virulence trials using this SP preparation.

Virulence for chicken embryos

Usually at 12-hour intervals following the inoculation of embryonated eggs with the three variants, 50 percent endpoints were calculated. These values were then used for plotting the embryo death curves for each variant, as shown in Figure 14. It may be seen that both the LP and MP variants (L and M respectively) caused rapid embryo deaths, and were of approximately equal virulence. In contrast, a fifty percent endpoint was not reached with the relatively pure SP variant (S).

Figure 14 includes, in addition, the results (S-M) of a titration of a mixed population of MP and SP variants,

Figure 14. Comparative virulence of the Records variants for chicken embryos

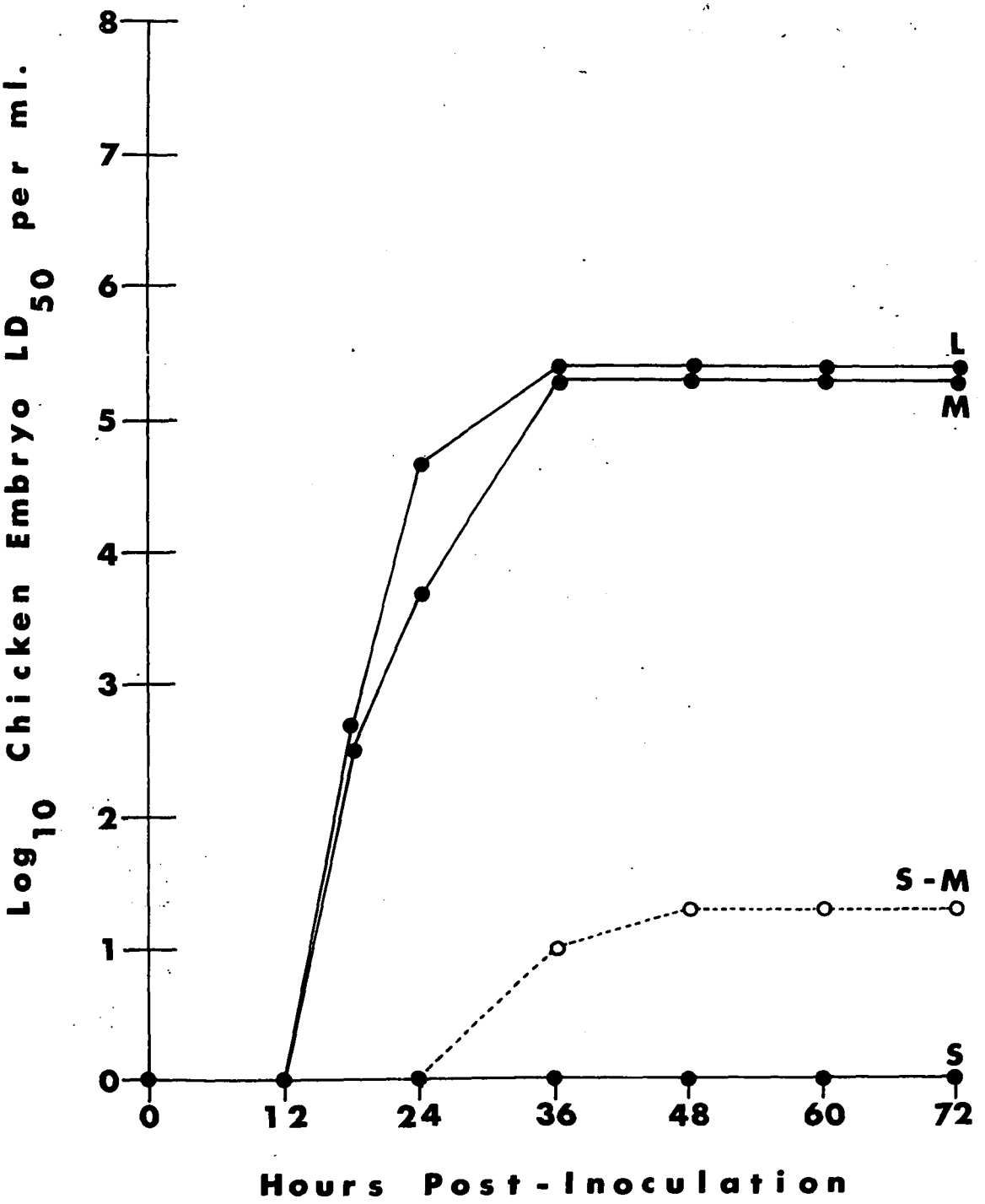
Letters indicate the following:

L. Pure LP variant

M. Pure MP variant

S. SP variant (1.6 percent impurity)

S-M. Mixed population (SP:MP = 1:1)


8
7
6
5
4
3
2
1
0
Log10 Chicken Embryo LD50 per ml.
L
M
S-M
S
0
12
24
36
48
60
72
Hours Post-Inoculation

present in approximately equal proportion. The total virus titer (MP and SP variants combined) equalled 2.4×10^8 PFU per ml. when this mixed population was titrated in CE cell cultures. Therefore the total virus given to the embryonated eggs was nearly equal to that given in the pure MP inoculum. From the pure MP titration, 1.0 LD_{50} was determined to contain $10^{3.88}$ PFU of MP variant. If given alone, and not mixed with the SP variant, 1.2×10^8 PFU should have equalled at least $10^{4.50}$ LD_{50} and not $10^{1.30}$ as actually titrated for the combined SP and MP variant inoculum. Clearly, the addition of an equal quantity of the SP variant was responsible for reducing the titer of the MP inoculum, and certainly did not enhance its infectivity.

Virulence for baby chicks

In Figure 15, time-death curves similar to those in Figure 14 are shown. These resulted from the determination of virulence of each Records variant for 12-hour-old chicks. By comparing curves M and L (MP and LP variants, respectively) with curve S (SP variant), it will be readily observed that a marked difference in virulence existed. Not only were there differences in the final endpoint titers, but also differences in the time at which the endpoints were reached.

As it appears in Figure 15, curve S is drawn so

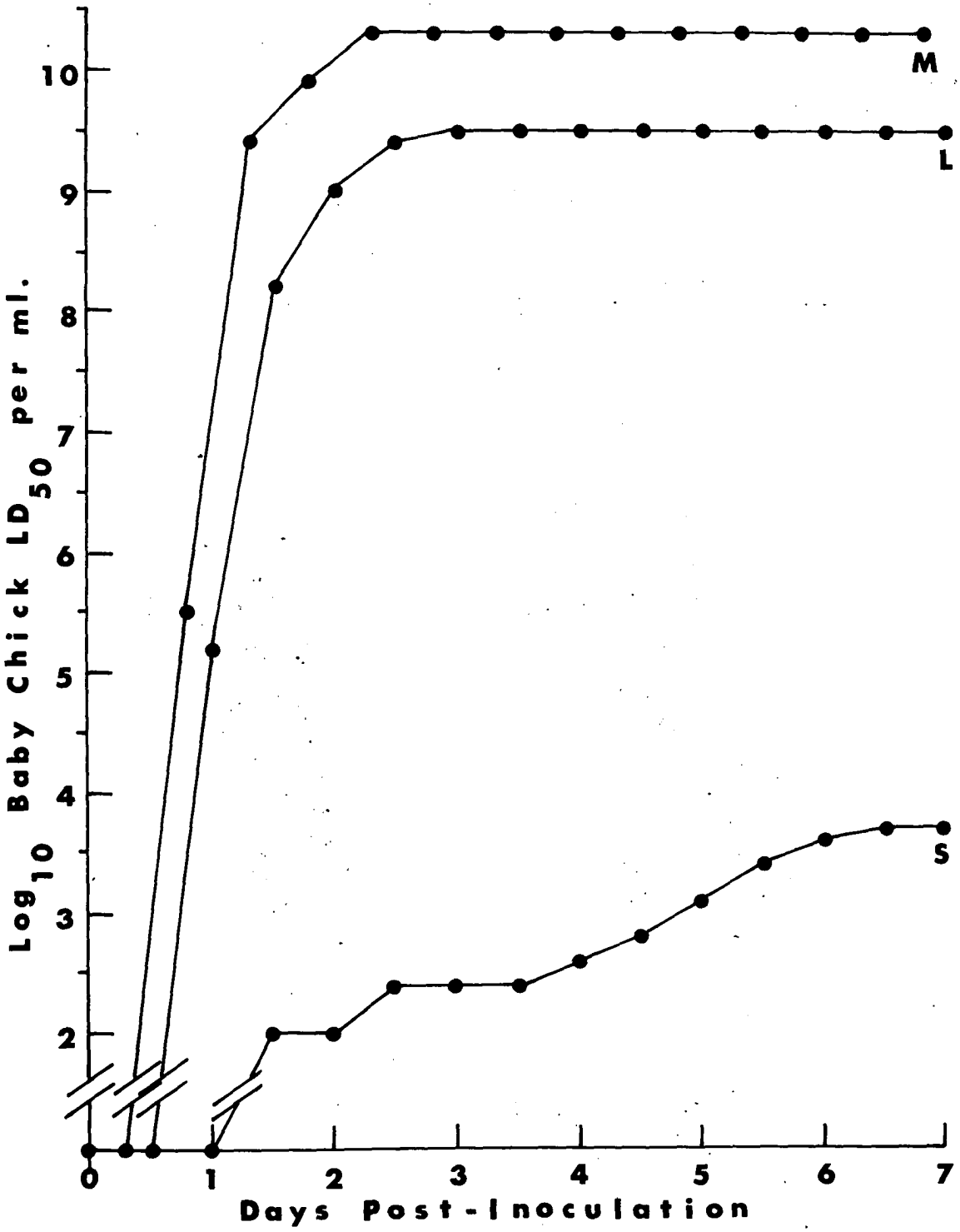
Figure 15. Comparative virulence of the Records variants for baby chicks

Letters indicate the following:

L. Pure LP variant

M. Pure MP variant

S. SP variant (1.6 percent MP impurity)


M
L
S
10
9
8
7
6
5
4
3
2
0
1
2
3
4
5
6
7
Log10 Baby Chick LD50 per ml.
Days Post-Inoculation

as to include the deaths of all chicks inoculated with the SP variant inoculum. This may misrepresent the results actually obtained and give the impression that the SP variant possessed greater virulence for baby chicks than was actually the case. Of significance once again is the fact that the SP inoculum contained 1.6 percent MP variant as an impurity. Reisolation of WEE virus from the brains of infected chicks yielded apparently pure MP variant, and no small plaques were detected by standard plaque assays of emulsified chick brain material.

Virulence for mice

Similar time-death curves resulting from titrations of the Records variants in weanling mice are graphed in Figure 16. Results from both intraperitoneal and intracranial routes of injection are included.

By the intracranial route, the relative virulence of the three variants was not strikingly different, especially if the respective titers (PFU per ml.) of the three inocula by cell culture titrations are considered.

A significant difference between the variants was detected however by the intraperitoneal titrations. By this route, the virulence of the LP and MP variants was

Figure 16. Comparative virulence of the Records variants for weanling mice

Letters indicate the following:

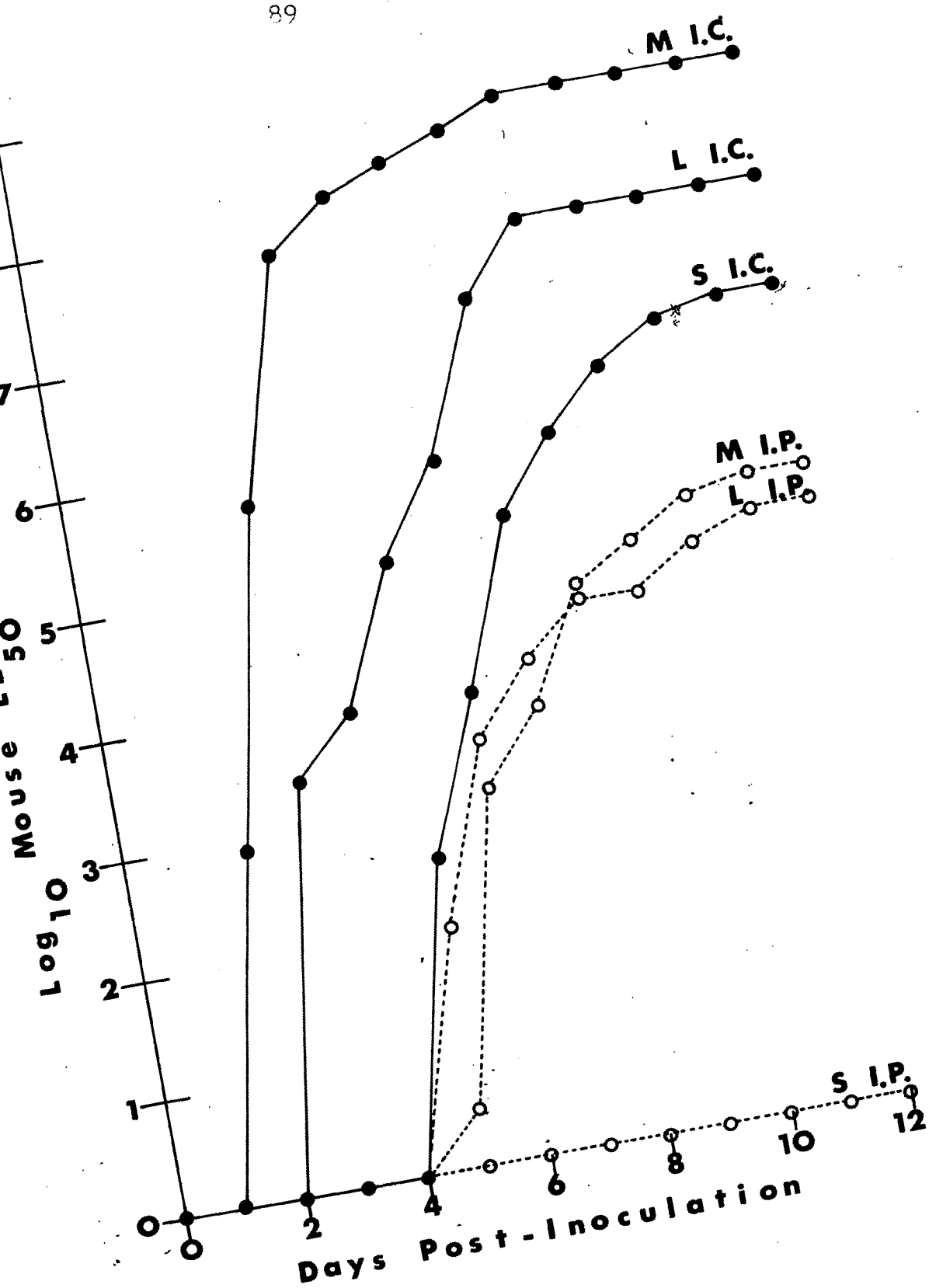
L. Pure LP variant

M. Pure MP variant

S. SP variant (1.6 percent MP impurity)

I.C. Intracranial route of injection

I.P. Intraperitoneal route of injection


M I.C.
L I.C.
S I.C.
M I.P.
L I.P.
S I.P.
Log10 Mouse LD50 per ml.
0
1
2
3
4
5
6
7
8
9
0
2
4
6
8
10
12
Days Post-Inoculation

still nearly identical, but the SP variant caused no deaths and no apparent signs of infection in mice, in spite of the presence of the MP impurity in the preparation used as inoculum.

Summary of results

For a better analysis of the results obtained in the virulence trials, they are again presented in composite form in Figures 17 and 18. In both figures, the final titer of each variant in each host may be directly compared with its respective cell culture titer.

It should be noted in Figure 17, that the titer of the SP variant in day-old chicks is again graphed as if it alone had been responsible for all of the deaths observed. There is reason to believe that the SP variant, if it could be completely purified, would possess little if any virulence for baby chicks. At least some of the deaths indicated were due to the presence of the MP impurity in the SP variant inoculum.

Comparison of Antigenic Properties[a]

Identification of the Records variants

Anti-LP and anti-WEE typing serum were found to have nearly the same virus neutralization titers (see Figure 19) when each was used in neutralization tests with the three

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[a]Complete data may be found in the Appendix.

Figure 17. Comparative titers of the Records variants determined by parallel titrations in cell cultures, baby chicks and chicken embryos

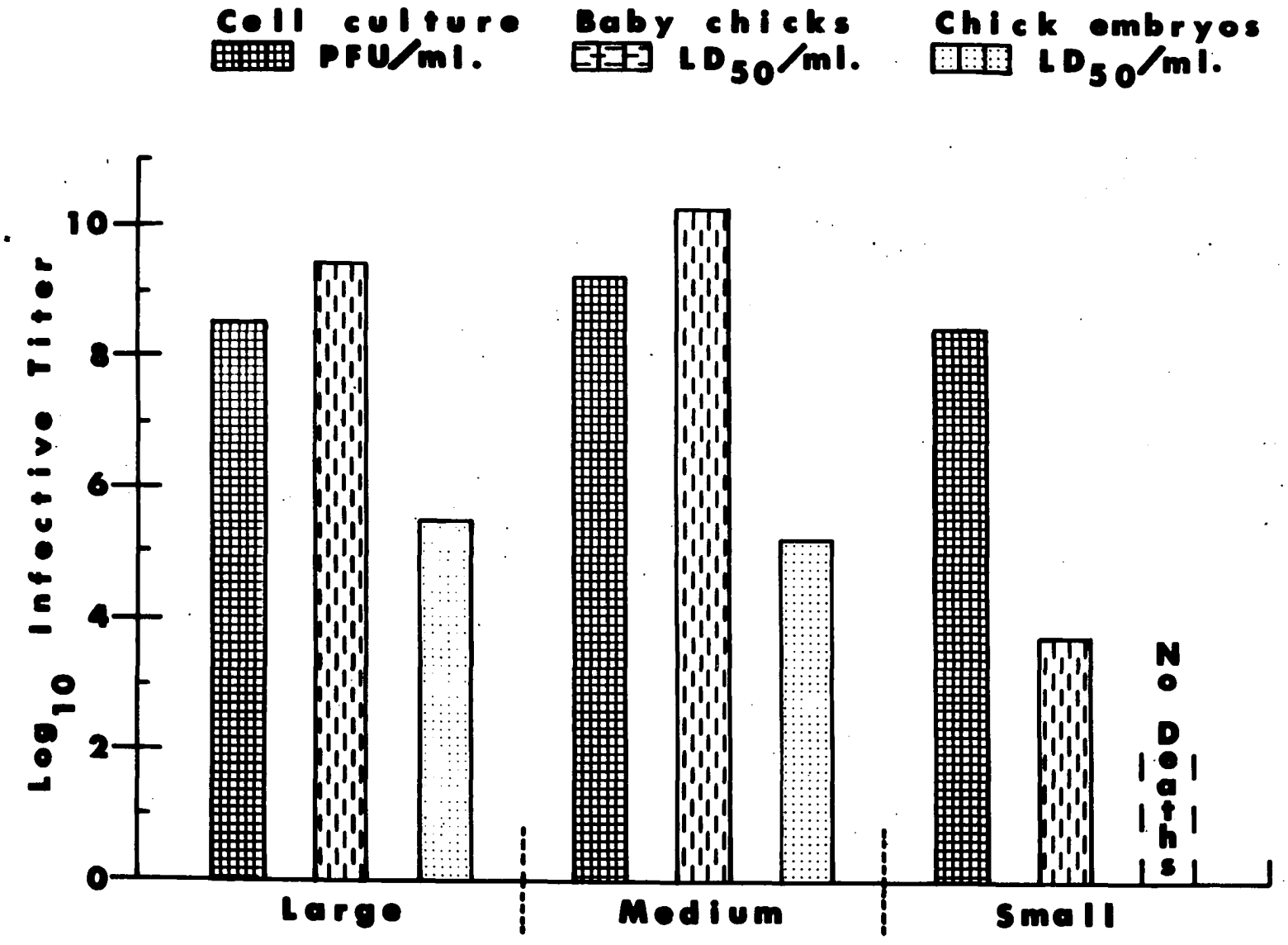

Cell culture
PFU/ml.
Baby chicks
LD50/ml.
Chick embryos
LD50/ml.
Log10 Infective Titer
10
8
6
4
2
0
Large
Medium
Small
No Deaths

Figure 18. Comparative titers of the Records variants determined by parallel titrations in cell cultures and in mice by intracranial (I.C.) and intraperitoneal (I.P.) routes

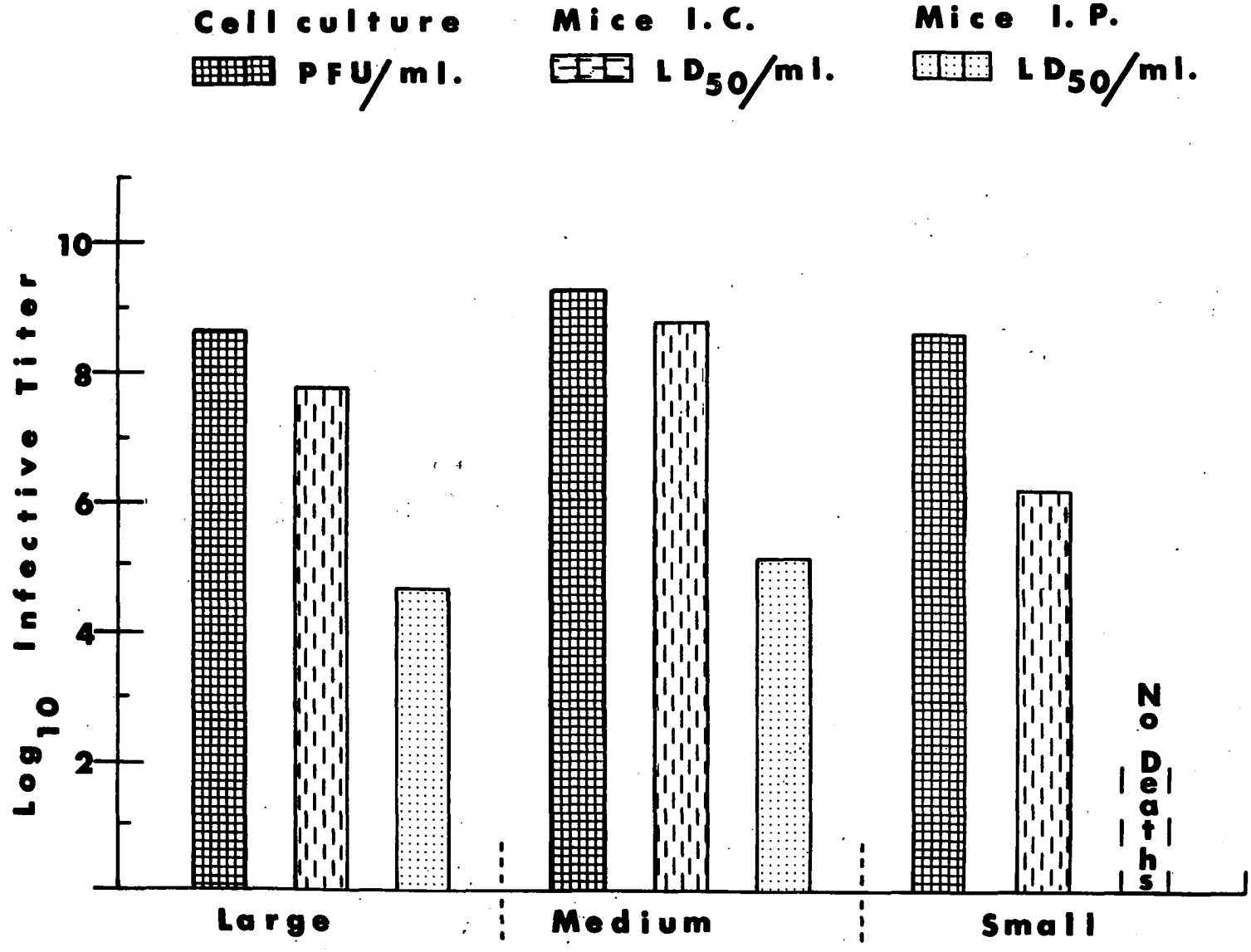
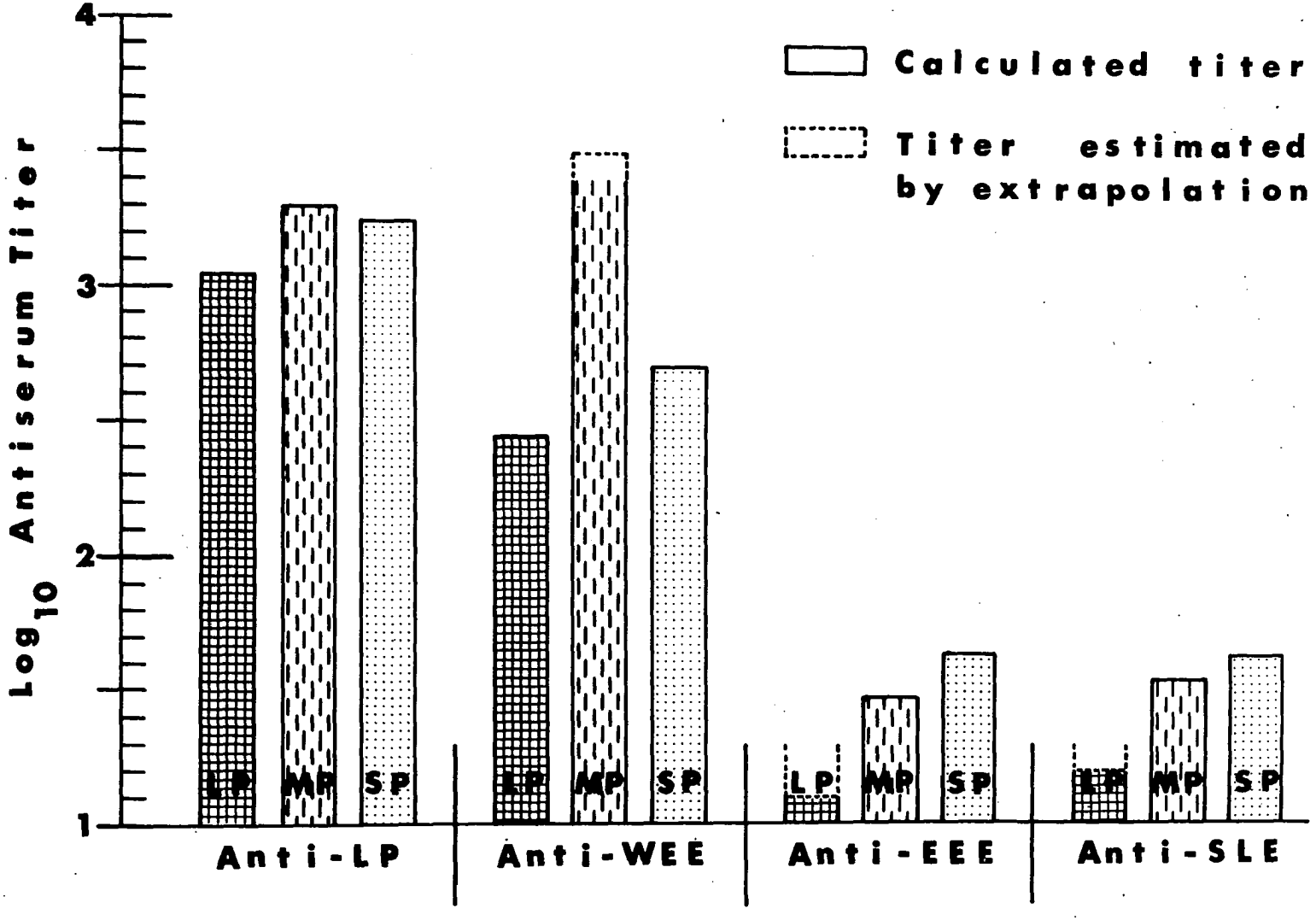

Cell culture
PFU/ml.
Mice I.C.
LD50/ml.
Mice I.P.
LD50/ml.
Log10 Infective Titer
10
8
6
4
2
No Deaths
Large
Medium
Small

Figure 19. Neutralization of the Records variants by an anti-LP serum from chickens, and by three arbovirus typing sera


Log10 Antiserum Titer
4
3
2
1
Calculated titer
Titer estimated by extrapolation
LP
MP
SP
Anti-LP
Anti-WEE
Anti-EEE
Anti-SLE

Records variants. The results presented in Figure 19 also indicate that there was a limited cross-reaction between the variants and the anti-EEE and anti-SLE typing sera. However, since all three variants were neutralized at significantly higher titers by the two homologous sera, they were all identified as WEE virus.

Reciprocal cross neturalization tests

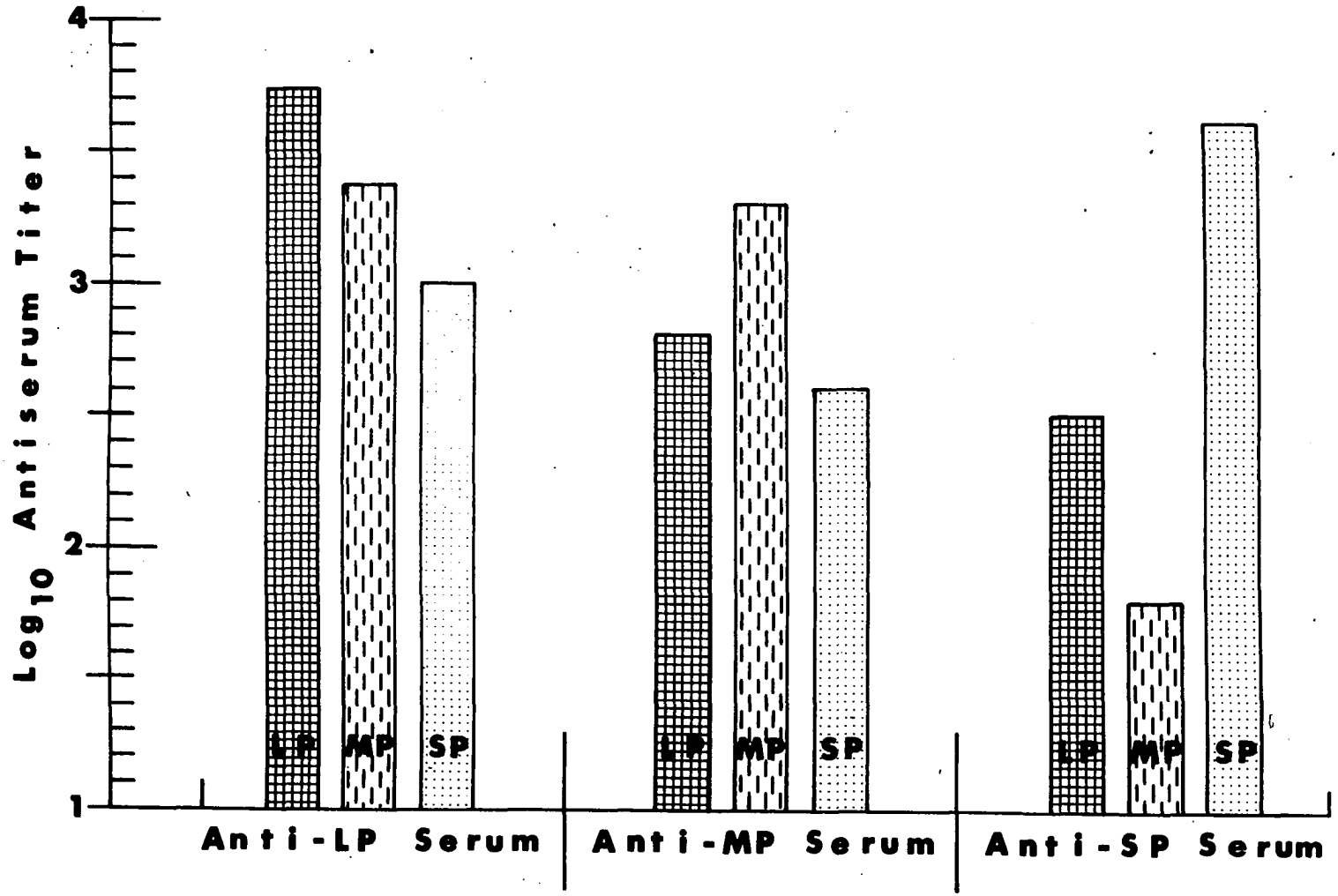
Neutralization tests were performed with each Records variant using anti-LP, anti-MP and anti-SP antisera prepared by immunization of guinea pigs. The test results were derived by accumulating and averaging the data from two replicate experiments.

It may be seen in Figure 20 that each antiserum exhibited its highest titer when tested against its homologous plaque-type antigen. Thus a degree of plaque-type specificity was possessed by each. This was especially true in the case of the anti-SP serum, since marked differences in its titer resulted from tests with the SP variant in comparison to those with the MP and also the LP variant. In contrast, the greatest degree of cross-reactivity was demonstrated with the anti-LP and anti-MP serum samples.

Hemagglutination (HA) tests

Propagation of WEE virus in CE cell cultures was apparently a quite satisfactory method of producing an

Figure 20. Reciprocal cross neutralization of the Records variants by guinea pig antiserum


Log10 Antiserum Titer
4
3
2
1
LP
MP
SP
LP
MP
SP
LP
MP
SP
Anti-LP Serum
Anti-MP Serum
Anti-SP Serum

HA antigen. The only difficulty encountered in the preparation of cell-culture-derived antigens was caused by traces of bovine serum which remained in the antigens if cell cultures were not thoroughly washed free of growth medium (containing serum) before the viruses were inoculated. These traces of serum markedly inhibited hemagglutination of chick erythrocytes by the three Records variant antigens especially at the low antigen dilutions. In the absence of the bovine serum inhibitor, readable and reproducible HA test results were readily obtained.

Using this method of antigen preparation, there was apparently no advantage to be gained by treating the virus preparations with Genetron (trifluorotrichloroethane) as suggested by Karabatsos (26, 27). Nearly identical test results were obtained with each of the antigens regardless of whether Genetron treatment had or had not been used. Apparently Genetron treatment was of no value in the attempted removal of bovine serum residues from those antigen preparations in which it was present.

Erythrocytes obtained from day-old chicks were used with satisfactory results. Under the same test conditions, cells from adult chickens or from sheep were not agglutinated. Goose erythrocytes were not readily available and were not used.

An unexplained result was observed in one experiment in which sodium dextran sulfate (100 micrograms per ml.) was added to the BBS diluent. If present by themselves, neither virus nor dextran sulfate caused hemolysis of chick erythrocytes, but quickly hemolyzed cells in tubes where the dextran sulfate and virus combination was present. With a constant concentration of dextran sulfate, progressively less hemolysis was observed with increasingly higher virus dilutions.

By changing the concentration of the phosphate buffers in the diluent used for the erythrocytes (PBS), the final pH of the test could be varied between 5.5 and 6.3, and the effect of pH on HA titers observed. The titers of the Records variants changed only by one or two dilutions throughout this pH range, but the highest titers were observed at pH 6.0 for each variant.

Table 7 contains typical HA test results, with the titration endpoints shown for each variant. When calculated, the PFU/HA ratios ranged from 4.4×10^5 to 7.5×10^6, with no consistent differences between the variants detected.

Hemagglutination-inhibition tests

HI tests were performed with both the 14-day and 35-day guinea pig antisera with results as shown in Tables 8, 9 and 10 for the three Records variants.

Table 7. Hemagglutination test results

Tube number:	1	2	3	4	5	6	7	8	9	10	11	12
Dilution:[a]	10	20	40	80	160	320	640	1280	2560	5120	10240	20480
Antigen:												
LP	+[b]	+	+	+	+	+	-[c]	-	-	-	-	-
MP	+	+	+	+	+	+	+	+	±	-	-	-
SP	+	+	+	+	+	±	-	-	-	-	-	-
Control	-	-	-	-	-	-	-	-	-	-	-	-

[a]Reciprocal of antigen dilution in BBS, pH 9.0, prior to addition of cells.

[b](+) = Complete hemagglutination.

[c](-) = No hemagglutination.

Table 8. Results of HI test using LP antigen

Tube number:	1	2	3	4	5	6	7	8	9	10	11	12
Dilution:[a]	20	40	80	160	320	640	1280	2560	5120	10240	20480	40960
Antiserum:												
LP 14	-[b]	-	-	-	-	-	-	+[c]	+	+	+	+
MP 14	-	-	-	-	-	-	+	+	+	+	+	+
SP 14	-	-	-	-	-	-	+	+	+	+	+	+
Control 14	+	+	+	+	+	+	+	+	+	+	+	+
LP 35	-	-	-	-	-	-	-	+	+	+	+	+
MP 35	-	-	-	-	-	-	-	+	+	+	+	+
SP 35	-	-	-	-	-	-	+	+	+	+	+	+
Control 35	+	+	+	+	+	+	+	+	+	+	+	+

[a]Reciprocal of serum dilution in BBS, before addition of virus and cells.

[b](-) = Hemagglutination inhibited.

[c](+) = Complete hemagglutination.

Table 9. Results of HI test using MP antigen

Tube number:	1	2	3	4	5	6	7	8	9	10	11	12
Dilution:[a]	20	40	80	160	320	640	1280	2560	5120	10240	20480	40960
Antiserum:												
LP 14	-[b]	-	-	-	-	-	-	-	-	+[c]	+	+
MP 14	-	-	-	-	-	-	-	-	-	+	+	+
SP 14	-	-	-	-	-	-	+	+	+	+	+	+
Control 14	+	+	+	+	+	+	+	+	+	+	+	+
LP 35	-	-	-	-	-	-	-	-	+	+	+	+
MP 35	-	-	-	-	-	-	-	-	+	+	+	+
SP 35	-	-	-	-	-	-	+	+	+	+	+	+
Control 35	+	+	+	+	+	+	+	+	+	+	+	+

[a]Reciprocal of serum dilution in BBS, before addition of virus and cells.

[b](-) = Hemagglutination inhibited.

[c](+) = Complete hemagglutination.

Table 10. Results of HI test using SP antigen

Tube number:	1	2	3	4	5	6	7	8	9	10	11	12
Dilution:[a]	20	40	80	160	320	640	1280	2560	5120	10240	20480	40960
Antiserum:												
LP 14	-[b]	-	-	-	-	-	-	+[c]	+	+	+	+
MP 14	-	-	-	-	-	-	±	+	+	+	+	+
SP 14	-	-	-	-	-	-	-	-	±	+	+	+
Control 14	+	+	+	+	+	+	+	+	+	+	+	+
LP 35	-	-	-	-	-	-	+	+	+	+	+	+
MP 35	-	-	-	-	-	-	+	+	+	+	+	+
SP 35	-	-	-	-	-	-	+	+	+	+	+	+
Control 35	+	+	+	+	+	+	+	+	+	+	+	+

[a]Reciprocal of serum dilution in BBS, before addition of virus and cells.

[b](-) = Hemagglutination inhibited.

[c](+) = Complete hemagglutination.

From a general comparison of HI titers of the antisera, it may be seen that the 35-day sera were in some cases even lower titered than the earlier 14-day samples. Also, as might be expected, there was some loss of specificity in the antisera collected later in the course of immunization.

In general, the HI test results provided a less sensitive comparison of the antigenic differences between the Records variants than did the cross-neutralization test. Only in the case of the MP and SP variants was a significant difference observed. Anti-MP serum inhibited hemagglutination by the homologous antigen at an antiserum dilution of 1:5120, but inhibited the SP variant only as high as a 1:640 dilution. A four-fold difference in the titers was seen in the 35-day antiserum. In the reverse situation, the early SP antiserum did possess a four-fold difference in HI titer between test results with the homologous and MP hemagglutinins. This difference in titer was not apparent however, in the 35-day sample.

Significant differences between the LP and MP antigens or between the LP and SP antigens were not apparent in the HI test results.

DISCUSSION AND CONCLUSIONS

Plaque Diameter Comparisons

Recent WEE isolates

Western equine encephalomyelitis virus populations, as they are isolated from naturally infected hosts, produce predominantly large plaques. This conclusion is based on the results of this study, in which seven WEE isolates from three different hosts and from three different states were all found to have mean plaque diameters very closely resembling that of the LP Records variant. A similar conclusion was reached by Marshall *et al.* (34) after their study of the plaque-size properties of six WEE isolates obtained in California, in 1958.

However, it may also be concluded, as did Ushijima *et al.* (57), that a degree of plaque-size heterogeneity exists in field strains of WEE virus. In the present study, medium-type plaques were observed during the standard plaque titrations of nearly all of the isolates. A possible reason for the failure to observe small plaques during the field strain titrations will be a topic for later discussion.

The Records strain

It has been shown that, after years of serial chicken embryo passage, the Records strain produced plaques which were apparently all in the medium size range. Judging by the plaque characteristics of the Records strain, it might be concluded that serial chicken embryo passage had selected for the MP variant from among a population which on initial isolation (like most field strains) could conceivably have been predominantly LP in character.

However, Marshall et al. (34) have suggested serial passage in chicken embryos as the only means of preserving the LP characteristic of wild strains of WEE virus. However, they did report the observance of medium-size plaques after chicken embryo passage of several strains of WEE virus. Thus their statement actually means that the mean plaque diameters of wild strains were not observed to decrease as rapidly during chicken embryo passage as they did in several other hosts.

Brown and Packer (4) reported that small plaques (2.0 to 5.0 mm. in diameter) became the predominant plaque type after 5 serial chicken embryo passages of a partially purified LP population. But, as indicated by the inclusion of the actual numerical values for the range in plaque diameter, medium-type plaques had actually been observed.

It was only after additional cloning experiments that a true SP variant (with typical plaques 0.5 to 2.5 mm. in diameter) was recognized within the Records strain.

Derivation of the Records variants

It is not known with certainty whether the LP and SP Records variants resulted from mutations of a relatively unstable MP variant, or whether they only appeared in the Records strain as a result of host selection from among a heterogenous population. The evidence which has thus far been accumulated, does however, strongly favor the hypothesis that the Records strain originally existed as a heterogenous population of at least the large and the medium plaque-size variants. Unless a minor component of a virus populations produces plaques which approximate nearly 1 percent of the total number, their presence is difficult to detect by plaque titrations. The medium plaque-type majority could therefore have masked the presence of the LP type until, as a result of host selection during guinea pig kidney cell culture passages, the relative proportion of the LP variant was increased.

Many research groups (5, 22, 30 and 45) have reported the observation of host-selection phenomena during the serial passage of WEE virus populations. It is probably then, not just coincidental that the LP and SP variants were first detected after serial passages of the predominantly

MP Records population in different host species. Circumstantial evidence indicates that serial passage in guinea pig kidney cell cultures may have been responsible for the appearance of the LP variant in one sample of the Records strain. There is also evidence that serial passage of another sample of the same virus in the brains of weanling mice was associated with the initial detection of the SP variant.

Host-selection phenomena are possible, of course, only when a mixed population is used for serial passage. When either the LP or MP variant exists as a plaque-purified population, they can be serially passed in CE cell cultures without detectable change in their mean plaque diameters. Both variants thus appear to be rather stable genetically. The changes in the plaque-size characteristics of SP populations which have been observed during serial passage can be attributed to their existence in an impure state. The significantly higher rate of accumulation of the MP variant following mixed SP-MP cell culture infection is likely a major factor governing the rapidity with which the MP variant becomes the major component of cell-culture-propagated populations. However, the questionable genetic stability of the SP variant might well be another factor contributing to the rapidity of this change.

Since replication of the MP variant is selectively favored in CE cell cultures, it is not at all surprising that the attempts to selectively clone the SP variant from amidst a mixed SP-MP population have met with considerable difficulty. One might suspect that serial intracranial passage in mice, if carried out at limiting dilution, might prove to be a more efficient method of purifying a predominantly SP population. At least in this host, replication of the SP variant would be selectively favored. From this viewpoint, it is interesting to consider the plaque properties of the Olitsky strain of WEE virus, as reported by Marshall et al. (34). The Olitsky strain has undergone innumerable serial mouse brain passages, and not surprisingly produces predominantly small plaques. But Marshall's group detected larger plaques (probably the MP variant) which comprised about 2 percent of the total number counted from Olitsky strain titrations. These results suggest that even serial mouse-brain passage would not result in a pure SP variant population. One might be led to speculate as to why the results of this study and that of Marshall and his coworkers might agree so closely with respect to the actual percentage of larger plaques detected in a SP population on its initial titration in CE cells. The figure of approximately 2 percent impurity has been independently determined as a result of both studies.

Could this figure of 2 percent be associated with a rather constant but relatively rapid back-mutation rate of the SP variant?

Field strains of WEE virus apparently do not produce small plaques when titrated by the standard plaque assay procedure. This fact, along with others, might suggest that the SP variant is strictly a laboratory curiosity which represents the neuro-adapted form of WEE virus. It lends added significance to the initial observation of the SP variant in samples of the Records strain only after serial mouse-brain passage. The SP variant probably differs genetically from the other variants, but represents a relatively unstable form. Host selection during intracranial passage in mice may be responsible for the perpetuation of this genotype in the laboratory, although even in this host, a rapid back-mutation rate might never allow it to exist in pure form. Its relatively rapid back-mutation rate, coupled with preferential host selection for the other variants and its relatively low peripheral virulence may all be contributing factors which might explain why the SP variant is seldom if ever isolated from naturally infected hosts.

Characteristic plaque size of the Records variants

The most confusing aspect of the literature dealing with WEE virus plaque-size variants is the variety of

environmental conditions used by the different research groups for plaque comparisons. These differences make correlation of the results reported by the different groups especially difficult, and may be primarily responsible for the apparent discrepancies found in the literature. For this reason, it will again be stressed that all plaque comparisons reported in this study were made under environmental conditions that were strictly controlled.

The values shown in Figure 2 of the preceding section may be taken as the most typical plaque diameter range and mean for each Records variant as obtained by the standard plaque assay procedure. It should also be re-emphasized that the classification of the Records strain of WEE virus into three plaque-size variants was not arbitrarily done. It can be shown statistically that 99 times out of 100, the plaques found in each classification could only have been produced by virus particles with significantly different properties. Therefore, the plaque diameter variation being studied was directly attributable to differences in the Records variants, and not due to environmental conditions.

Effects of agarose overlays

Even though the large and medium type plaques resembled each other more closely under agarose overlays than they did by the standard plaque assay procedure, a significant difference in their mean plaque diameters

could still be statistically demonstrated. With agarose overlays, the plaques produced by the MP variant were significantly larger than those of the LP variant, results which were in direct contrast to those obtained under Noble agar. But, these differing results are explainable.

It has been shown that the MP variant, of the two being discussed, is more susceptible to the inhibitory effects of sulfated polysaccharide. With a comparatively lower level of polysaccharide sulfate inhibitor in agarose, the full growth potential of medium-type plaques is then more likely expressed. In CE cell cultures maintained with a fluid medium (in the absence of agar) the MP variant has a demonstrably higher rate of accumulation. Since the rate at which MP virus particles are made available for the infection of adjacent cells is slightly greater than for the LP variant, the plaques produced by the MP variant evidently achieve a slightly greater diameter in the specified 48-hour time interval allowed for development.

In a similar way, one can explain the results observed with the SP variant. Under Noble agar overlays, development of small plaques is limited by the low growth potential of the SP variant in CE cells, and even further limited by the susceptibility of the SP variant to polysaccharide sulfate inhibition. When the inhibitor is present in lesser concentration, as in agarose overlays, the low rate

of accumulation of the SP variant in the cultures still remains as the principal factor limiting plaque development. Although they are perhaps equally inhibited by the sulfated polysaccharide, the SP and MP variants still differ markedly in their respective rates of accumulation. Thus, the difference between small and medium plaques is even greater under agarose than when these two variants are titrated by the standard plaque assay procedure.

It might therefore be concluded that the characteristic plaque size of each variant under agarose overlays is in direct proportion to the rate of accumulation of newly synthesized virus in the cell cultures. If this conclusion is correct, it serves as excellent corroboration of the results obtained in the growth rate studies. It also agrees with the observation that the MP variant is most likely to be selected from a mixed population of WEE virus during serial passage in CE cell cultures or in embryonated eggs.

Effects of inhibitors added to agarose overlays

Colón and his collaborators (9, 10 and 11) reported the effects of sulfated polysaccharide from agar after studying only a susceptible or SP variant of WEE virus. Other groups have demonstrated the effects of the inhibitor only by indirect means. For example, DEAE dextran used in

Noble agar overlays by Ushijima *et al.* (57) was apparently responsible for significantly increasing the diameter of their SP variant by blocking the action of the agar inhibitor. Since the relative degree of increase of the small plaque type was greater than that of the large, these workers were able to suggest that a difference in inhibitor susceptibility existed between the two variants.

In the current study, the use of agarose overlays provided an opportunity to directly determine the effects of various inhibitory polysaccharides on the plaque diameter of each Records variant. The results indicate that all three variants are susceptible to the inhibitory effects of sulfated polysaccharide preparations, and that inhibitors in the overlay actually do cause a marked decrease in the size of the plaques which each produces. Of the three variants, the LP was the least affected by the addition of crude inhibitor preparations from Noble or Bacto agars. This lower susceptibility of the LP variant is the principal factor responsible for its production of large plaques under Noble agar overlays. Yet, in the concentration used, dextran sulfate caused a reduction in the size of the large plaques to the point where they became indistinguishable from those produced by the MP variant under the same conditions. This indicates that the LP variant is not totally resistant to the effects of sulfated

polysaccharide, and that the difference between it and the MP variant in this respect is only relative.

Effects of cysteine and lactalbumin hydrolysate added to skim milk overlays

Under skim milk-Noble agar overlays, all three Records variants produced smaller-than-normal plaques. Addition of cysteine to the overlay medium caused an increase in the mean plaque diameter of each variant, but only after the addition of lactalbumin hydrolysate (LAH) did the resulting mean plaque diameters approximate those normally obtained by the standard plaque assay procedure. It has been demonstrated that this plaque-size increase was not the result of a correction of a nutritional deficiency of the skim milk overlay medium. Instead, it apparently resulted from a decrease in the susceptibility of the variants to inhibition by the sulfated polysaccharide of Noble agar. Under skim milk-agarose overlays, larger plaques developed even without the addition of cysteine or LAH.

It may be concluded that the characteristic plaque diameters of the three Records variants under the standard plaque assay conditions are dependent on the presence of the sulfated polysaccharide of Noble agar and possibly on the cysteine of the LAH which are both customarily used in the overlay medium.

It is also readily apparent that the resistance to polysaccharide sulfate inhibition of the LP variant, in comparison to the other two, is more significantly enhanced by the presence of LAH in the overlay. The principal difference between the LP and MP Records variants is marked by this difference in their resistance to plaque inhibition by sulfated polysaccharide. In the absence of cysteine of LAH, even this distinction is no longer demonstrable.

According to Wallis and Melnick (58), a 0.5 percent solution of LAH would be 0.5 millimolar with respect to cysteine. In their studies with the enteroviruses, they apparently assumed that the protective effect of LAH was principally due to its cysteine content. The three Records variants produced larger plaques in the presence of 0.5 percent LAH than in the presence of the 2.0 millimolar concentration of cysteine (4 times the equivalent initial cysteine concentration). Due to the characteristic instability of cysteine in aqueous solution, it is difficult to determine whether or not some other component of LAH may also have had a protective effect for the Records variants in the current study.

Effects of bovine serum

It has been demonstrated, that under either Noble agar or agarose overlays, bovine serum in 5 percent concentration

slightly inhibits the development of medium-size plaques. This result is in close agreement with other effects of bovine serum observed during the current study. It is known that traces of bovine serum, if present in HA antigens, will markedly inhibit hemagglutination of chick erythrocytes by the Records variants. During propagation of the MP variant in CE cell cultures, higher virus titers were obtained, and cytopathic effects were observed to occur more rapidly if bovine serum was omitted from the fluid maintenance medium. Thus, as is shown by its inhibitory effect on MP development, bovine serum probably contains some component which is capable of direct interaction with the MP variant and therefore reduced the effective concentration of free virus particles during CE cell culture infection.

The inhibitory effects of serum are more readily apparent with the MP variant than with the other two. As indicated by the decrease in its mean plaque diameter caused by the addition of bovine serum to agarose overlay medium, the LP variant is also susceptible to the inhibitory effects of serum. Why then, was an opposite effect observed under Noble agar overlays when bovine serum was added? It is possible that the presence of serum might enhance the resistance of the LP variant to the inhibitory effect of sulfated polysaccharide, in the same manner as

has already been demonstrated for cysteine and lactalbumin hydrolysate. This enhancing effect may likely be greater than the inhibitory activity of serum, and a net reduction in mean plaque diameter was therefore detectable only under agarose overlays where the enhanced resistance to sulfated polysaccharide was of no consequence in plaque-size determination. This enhancement was not seen in the case of the MP variant under Noble agar since perhaps serum (like LAH) only partially protects the MP variant against inhibition.

Since the rate of accumulation of the SP variant in CE cell cultures is already limited, any inhibitory effects of serum on its plaque development might possibly remain undetected. However, if any significant effect of serum was observed, it was a slight enhancement, both under Noble agar and agarose overlay media. It is possible that the SP variant is less likely to react with bovine serum than are the other two variants. During other experiments there was additional evidence in support of this conclusion. On several occasions, it was observed that the SP character of a relatively pure SP population was more readily maintained during passage in CE cell cultures if 5 percent bovine serum was added to the fluid maintenance medium. In the absence of serum, the resulting populations often contained a higher proportion of the MP variant. This observation suggests that of the two, the MP variant is

more strongly inhibited by bovine serum. Its relative non-reactivity with bovine serum may be still another distinctive property of the SP Records variant.

Virus and sulfated polysaccharide interaction

A number of research groups have studied the reactions of viruses with the inhibitory sulfated polysaccharides. Most studies have resulted in conclusions similar to those of Colón et al. (9, 10 and 11) who have investigated the effects of agar extracts and dextran sulfate on several arboviruses. Although an inhibitor-host cell interaction could not be completely disproven, Colón and his coworkers concluded that a direct interaction between sulfated polysaccharide and the virus particle was the most likely cause of inhibition of WEE virus plaque development.

Nahmias et al. (43) investigated the effects of a group of synthetic and biological polyanions on herpes simplex virus plaque formation. In order to possess inhibitory activity, the compounds they studied needed to be of a minimum molecular size or degree of polymerization. The activity of these inhibitors was clearly a function of their sulfate ion content and not solely due to negative charge, since hyaluronic acid (which contains negatively charged carboxyl groups) was found to be inactive. The polysaccharide structure itself was unnecessary, as evidenced

for example, by the inhibitory effect of polyvinyl alcohol and polystyrene sulfonate.

Similar conclusions can readily be made from the results of this study with the Records variants. The decreased sulfate ion content of agarose in comparison to Noble agar is probably responsible for the significantly decreased inhibitory activity of agarose overlays. Furthermore, inhibition of WEE virus plaque development was directly attributable to the addition of sulfated polysaccharide, either in the form of agar extracts or as dextran sulfate, to agarose overlays.

The principal difference between the LP and MP Records variants is associated with their apparent difference in susceptibility to plaque-inhibition by sulfated agar polysaccharide. It might initially be assumed that some specific property of the LP variant might account for its relative nonreactivity with the sulfate ion of agar polysaccharide.

Other research workers have actively searched for differences in the physical and chemical properties of inhibitor-susceptible and inhibitor-insusceptible variants. Working with VEE virus, Heydrick et al. (23) detected differences in banding patterns between LP and SP variants in sucrose density gradients, and also a difference in the ratios of soluble to insoluble lipids. From a study of the amino acid composition of proteins obtained from three

plaque-size variants of encephalomyocarditis virus, Moscarello and Kaighn (40) detected several minor differences. However, the detectable differences reported by either group were probably not sufficient to account for a significant difference in the reactivity of these variants with the sulfate ion of polysaccharide inhibitors.

According to Moscarello and Kaighn, the remarkably low concentration of sulfur-containing amino acids in encephalomyocarditis virus proteins was perhaps the most interesting feature of their results. They commented that low levels of methionine, cystine and cysteine seem to be a universal property of all animal virus proteins which have thus far been analyzed.

Possible significance of the protective effect of cysteine

It has been demonstrated that there is only a relative difference between the LP and MP Records variants in their susceptibility to the inhibitory effects of sulfated polysaccharide. There was evidence that the relatively greater resistance of the LP variant was overwhelmed by the addition of dextran sulfate to agarose overlays. Under skim milk overlays, devoid of lactalbumin hydrolysate (LAH), there was virtually no difference in the susceptibility of these two variants to sulfated polysaccharide inhibition. It might therefore be concluded that the LP and MP variants are both capable of direct reaction with sulfate ion and that the LP

variant, of the two, is preferentially protected from entering into this reaction in the presence of LAH. Since cysteine shows a similar sparing effect, there is also reason to believe that this protective activity of LAH is at least in part related to its cysteine content. These latest findings may perhaps explain why the detectable differences in the physical and chemical properties of plaque-size variants might not satisfactorily account for a difference in their tendencies to react directly with sulfated polysaccharide.

A possible mode of action of cysteine and LAH

Protamine sulfate (4) or DEAE dextran (57), if added to Noble agar overlays, have also been reported to cause a demonstrable increase in the plaque size of WEE virus. A logical assumption might be that these polycations play a protective role by reacting with the negatively charged sulfate groups of the inhibitor, thereby preventing their reaction with the susceptible virus. The nondiscriminatory protective effects of DEAE dextran or protamine sulfate may thus be explained. A direct reaction between cysteine and the sulfated polysaccharide could also be postulated, however its occurrence would not readily explain the preferential protection of the LP variant as contrasted to the other two. Therefore, it might be suggested that cysteine instead exerts its protective influence by reaction

with the virus itself. If this is the case, it could also be assumed that the preferential protection of the LP variant is a result of a difference in its reactivity with cysteine or perhaps with a similar component of LAH.

This hypothesis is strongly supported by the observations of Wallis and Melnick (58) who have studied the protective effects of cysteine for the Pesascek echovirus. Not only is this LP echovirus variant protected against plaque inhibition by sulfated polysaccharide, it is also stabilized against heat-inactivation in the presence of cysteine. Clearly this latter effect must be due to the influence of cysteine on the virus itself, since heat-inactivation studies were carried out in the absence of sulfated polysaccharide.

Surely, additional studies of the reactions of cysteine and similar compounds with the Records variants might result in valuable information concerning the surface properties of these and similar infective agents.

Virulence Comparisons

Comparison of host sensitivity

The principal purpose of the virulence trials was to investigate possible differences in virulence which might exist among the three Records variants. This purpose was accomplished by parallel titrations of similar preparations of each variant in several different laboratory

hosts. However, before any general conclusions may be drawn, it is necessary to first consider the relative susceptibility of the various host species to WEE virus infection. This rating of the hosts according to their apparent sensitivity is more meaningful if the results of the SP variant titrations are initially disregarded. It was observed in this study, that the highest titration endpoints with both the LP and MP variants were reached after infection of ½-day-old chicks. In the order of decreasing sensitivity, one could further list the standard plaque assay procedure in CE cell cultures, intracranial injection of weanling mice, intra-allantoic infection of chicken embryos and finally, titrations in weanling mice by the intraperitoneal route. This rating of host sensitivity is coincidentally the same as the order in which they might be selected for use in the isolation of WEE virus from field specimens, since wild strains are also MP and LP in character.

Relative virulence of the Records variants

The results of the virulence trials demonstrate that there is little difference in the virulence of the LP and MP variants. In each of the laboratory hosts tested, nearly identical infective titers were obtained for these variants.

Significantly different results were obtained however, with the SP variant. Only in mice injected intracranially,

did the SP variant demonstrate virulence closely comparable to that of the other two. Despite the presence of the MP variant impurity, the SP variant preparation did not produce deaths of mice inoculated intraperitoneally or deaths of embryos after intra-allantoic injection of embryonated eggs. The deaths which did occur in the baby chicks after subcutaneous administration of the SP inoculum are thought to be due, at least in part, to the MP impurity which it contained. Since the MP variant was present in sufficient concentration to be detectable by cell culture titrations, it is not surprising that its presence was also detected by infection of baby chicks, an even more sensitive titration procedure. Thus, for the first time, it has been observed that there is a direct relationship between virulence and plaque-size characteristics of WEE virus.

The SP marker and virulence

The results of the virulence trials are closely comparable to those reported by Hearn et al. (20) and Hardy and Hearn (19) after their investigations with VEE virus. Also, Chamberlain et al. (5) have reported that the mouse-adapted Flemming strain of WEE virus (presumably also a SP strain) possessed decreased virulence for baby chicks, and Kissling (30) has noted that a loss of peripheral virulence results from serial intracranial passage of most arboviruses. Therefore, the observation of the

reduced peripheral virulence of the SP variant in this study was not unexpected, and was essentially a confirmatory finding. One main conclusion, supported by previous observations, may be drawn as a result of the virulence trials with the Records variants. It may be stated that the SP marker which appears during serial intracranial passage of a WEE virus population is visible indication that this population then possesses reduced virulence for many potential hosts.

Autoinhibition effects

The results of virulence trials in embryonated eggs indicated that embryos were partially protected against the lethal effects of MP infection in the presence of the less virulent SP variant. Additional evidence which suggests that the SP variant is capable of interfering with the replication of the MP variant was obtained by growth rate comparisons in CE cell cultures. The MP variant was observed to accumulate at a rapid rate in cultures after infection with a pure MP inoculum. But, its rate of accumulation was comparatively slower after a mixed MP-SP infection. While neither of the experiments was specifically designed to detect interference by the SP variant, the results strongly suggest that it did occur.

Dunayevitch et al. (18) observed similar interference effects caused by a variant of low pathogenicity found to

be a component of a field strain of WEE virus. The results of the current study with the Records variants lend support to their conclusion that the presence of a non-pathogenic variant in a mixed population of WEE virus may protect animals against the lethal effects of infection by the more virulent variant.

Stability of the SP variant

It has been shown that the relatively avirulent SP variant has been impossible to purify by selective cloning procedures. After only two serial passages in CE cell cultures with fluid maintenance medium, the MP variant again represents the majority of the virus population. From the results of titrations in embryonated eggs and in baby chicks, there is also evidence that, along with its change in plaque characteristics, this population also regains much of its original virulence. Failure to regain complete virulence might be accounted for by the interference effects just described, as long as the SP variant was also present in the population. It might therefore be concluded that this apparent instability of the SP Records variant is a highly undesirable property which would eliminate it from consideration as a possible live-virus vaccine strain.

The clone 15 variant currently being investigated as a potential live-virus vaccine was derived from the

mixed WEE virus population studied by Dunayevitch et al. (18). Although its plaque-size properties were not considered during the selective cloning of this variant from the original heterogenous population, Johnson[a] has stated that the clone 15 variant, in its current form, still produces a mixture of small and intermediate-sized plaques in chicken embryo cell cultures. Although the clone 15 variant resembles the SP Records variant in many of its properties, one must hope that it at least proves to be much more stable than the Records variant during serial passage experiments.

Comparison of Antigenic Properties

The SP marker

The results of serum neutralization tests and of hemagglutination-inhibition tests both indicate that the SP variant possesses antigenic properties significantly different from those of the other two Records variants. The degree of difference among the three variants is also suggested when the quantitative differences in antiserum titers are considered. It might appear, as a result, that the SP variant is more closely related

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[a]Johnson, H. N. The Rockefeller Foundation, Berkeley, California. Unpublished observations. Private communication.

in its antigenic structure to the LP variant, and more distantly related to the MP variant. And, of the three, the closest relationship is between the LP and MP variants.

Henderson and his collaborators (21, 22, 28 and 52) have demonstrated the antigenic heterogeneity of WEE virus populations, host selection of antigenic variants accompanying serial passage and differences in the replication rates of respective variants in CE cell cultures. A marked difference in the antigenic properties of newly isolated strains and mouse-passaged laboratory strains has also been detected, first by Olitsky et al. (45) and later by Karabatsos et al. (28). It may be surmised that the Olitsky strain, as later reported by Marshall and his coworkers (34), existed principally as the SP variant. These previously reported findings are all quite similar to the results of the current study. The close correlation of the results obtained by all these research groups clearly demonstrates that the SP marker may well be a significant indicator of change in the antigenic properties of WEE virus. Therefore, the observation of small plaques resulting from the titration of a population of WEE virus in CE cell cultures is an indication that this population has undergone significant changes, both in virulence and in antigenic properties, from its characteristic wild state.

The antigenic change which WEE virus populations are

observed to undergo during serial intracranial mouse passage has, in the past, confused the virologist who was attempting to identify newly isolated field strains. For example, the antigenic differences between a LP field strain and a SP laboratory strain might be nearly as great as the antigenic differences between the field strain and another arbovirus. The results of serological tests might be more easily interpreted if the antigenic properties of new isolates were compared with those of the plaque-purified LP variant, and not with those of mouse-propagated stock strains which are more likely to be predominantly SP in character.

Since hemagglutination-inhibition tests are most likely to be used for the initial identification of many new arbovirus isolates, a case might be made for the propagation of HA antigens in CE cell cultures rather than their propagation by intracranial passage in suckling mice. Satisfactory HA antigens can be produced in cell cultures by a method which does not require complex preparatory procedures. And since the use of a plaque-purified LP antigen is advisable, its purity could be more readily maintained and monitored by cell culture propagation procedures.

A discussion of the antigenic differences of the SP variant would not be complete unless there was an explanation

of why other investigators of WEE virus plaque-size variants failed to detect this apparent difference. In most of the other studies, reciprocal cross-neutralization tests were not employed. None of the other groups used guinea pig antisera. And, because of the apparent instability of the SP variant, the use of formalin-inactivated antigens in the current study was probably the principal reason for its success.

The LP and MP variants

The antigenic differences between the LP and MP variants appear to be less distinctly demonstrable. Additional and more definitive studies may be necessary before the full significance of these apparent differences can be assessed. Antibody absorption studies with each of the variants, patterned after those of Clarke (6, 7) with the group B arboviruses, would seem to be a logical approach to further investigation.

SUMMARY

Three plaque-size variants were selectively cloned from the Records strain of WEE virus after its serial passage in several different host species. They were designated as the LP (4 to 7 mm.), MP (2 to 5 mm.) and SP (0.5 to 2.5 mm.) Records variants. These characteristic ranges in plaque size were dependent on the presence of the sulfated polysaccharide of Noble agar and the presence of lactalbumin hydrolysate (LAH) in the overlay medium during a standardized plaque assay procedure in CE cell cultures.

The LP and MP Records variants have many properties in common. Each could be obtained in pure population by the selective cloning procedure. They both appear to be rather stable genetically and are of nearly equal virulence for many laboratory hosts. Normal bovine serum slightly inhibits infectivity and hemagglutinating activity of both. They differ only slightly in antigenic properties and there is perhaps a small difference in their growth rates during CE cell culture infection. Under agarose overlays which are known to be low in sulfated polysaccharide, their mean plaque diameters are quite similar.

The principal difference between the LP and MP Records variants is in their relative susceptibility to plaque inhibition by sulfated polysaccharide. The significantly greater resistance of the LP variant to reaction with

sulfated polysaccharide is primarily responsible for its production of large-type plaques under standard assay conditions. This resistance of the LP variant is attributable, at least in part, to the cysteine component of LAH which exerts a preferential protective effect on the LP variant. The possible significance of this finding is discussed.

Newly isolated field strains of WEE virus typically produce both large and medium type plaques. Small plaques were not detected during the titrations of seven new isolates, and were found in the Records strain only after its serial intracranial passage in weanling mice.

The SP Records variant, in comparison with the other two, possesses many distinguishing properties. It was not possible to prepare a pure SP variant population by selective cloning in CE cell cultures, a possible indication of genetic instability of this variant. In mixed infection, the SP variant apparently interfered with the replication of the MP variant. Of the three variants, the SP is perhaps the least reactive with normal bovine serum.

The SP variant accumulated in the maintenance medium of infected CE cell cultures at a rate significantly lower than the other two. This fact may account for its production of plaques significantly smaller than those of the other two variants under agarose overlays. An additional factor responsible for its production of even smaller

plaques under the standard plaque assay conditions is its suceptibility to inhibition by agar polysaccharide.

Of perhaps greater importance are the observations that the SP variant possesses markedly reduced peripheral virulence for several hosts, and is antigenically distinguishable from the other variants. These findings are compared and correlated with those of other investigators, and the significance of the WEE virus SP marker is discussed.

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APPENDIX

Table 11. Diameter of plaques produced by the Records strain and by the three Records variants under standard plaque assay conditions

Virus:	Plaque diameter (mm.) 1	2	3	4	5	6	7	8	9	10	Mean[a]
Records strain	-[b]	1[c]	24	50	12	-	-	-	-	-	3.9
LP variant	-	-	-	2	53	76	13	1	-	-	5.5
MP variant	-	2	22	29	2	-	-	-	-	-	3.7
SP variant	189	92	5	-	-	-	-	-	-	-	1.3

[a]Mean plaque diameter (mm.).

[b]None observed.

[c]Number of plaques.

Table 12. Comparison of the plaque diameters of seven recent isolates with those of the Records variants

Virus:	Plaque diameter (mm.) 1	2	3	4	5	6	7	8	9	10	Mean[a]
LP variant	-[b]	-	-	-	20[c]	35	12	1	-	-	5.9
MP variant	-	13	75	22	1	-	-	-	-	-	3.1
SP variant	150	21	-	-	-	-	-	-	-	-	1.1
65V-241	-	-	-	-	20	35	12	1	-	-	5.9
65V-796	-	2	-	-	6	8	3	1	-	-	5.6
65V-657	-	-	-	-	11	28	6	-	-	-	5.8
65V-692	-	3	-	2	28	12	2	-	-	-	5.1
65V-633	-	2	-	5	20	16	-	-	-	-	5.1
65V-629	-	1	4	5	22	37	1	-	-	-	5.3
E 2329	-	2	1	18	36	33	14	-	-	-	5.3

[a]Mean plaque diameter (mm.).

[b]None observed.

[c]Number of plaques.

Table 13. Plaque diameters of the Records variants under Noble agar or agarose overlays

Overlay:	Plaque diameter (mm.) 1	2	3	4	5	6	7	8	9	10	11	Mean[a]
LP variant												
Noble agar	-[b]	-	-	2[c]	53	76	13	1	-	-	-	5.7
Agarose	-	-	-	-	6	24	37	3	-	-	-	7.5
MP variant												
Noble agar	-	2	22	292	2	-	-	-	-	-	-	3.5
Agarose	-	-	-	-	-	3	4	13	17	15	3	8.9
SP variant												
Noble agar	189	92	5	-	-	-	-	-	-	-	-	1.3
Agarose	-	-	31	131	61	6	-	1	-	-	-	4.2

[a]Mean plaque diameter (mm.).

[b]None observed.

[c]Number of plaques.

Table 14. Effect on plaque diameters of inhibitory substances added to agarose overlays

Substance[a] added:	Plaque diameter (mm.) 0.5	1	2	3	4	5	6	7	8	9	10	Mean[b]
LP variant												
AE	-[c]	-	-	-	-	7[d]	10	8	17	1	-	6.9
NE	-	-	-	-	-	1	10	12	9	-	-	6.9
BE	-	-	-	1	12	23	8	1	-	-	-	4.9
DS	1	7	9	11	10	-	-	-	-	-	-	1.8
MP variant												
AE	-	-	-	-	-	3	4	9	14	2	-	7.3
NE	-	-	-	7	11	16	9	3	2	-	-	4.9
BE	-	-	7	-	18	14	-	-	-	-	-	3.2
DS	4	8	8	14	2	-	-	-	-	-	-	1.5
SP variant												
AE	-	-	-	-	-	29	61	37	-	-	-	5.1
NE	-	-	12	42	40	26	-	-	-	-	-	3.7
BE	-	21	71	17	-	-	-	-	-	-	-	2.0
DS	24	78	10	-	-	-	-	-	-	-	-	1.0

[a]Abbrevations are as follows: AE = agarose extract, NE = Noble agar extract, BE = Bacto agar extract, DS = dextran sulfate.

[b]Mean plaque diameter (mm.).

[c]None observed.

[d]Number of plaques.

Table 15. Plaque diameters of the Records variants under skim milk overlays of varied composition

Over-lay:[a]	Plaque diameter (mm.)													Mean[b]
	0.5	1	2	3	4	5	6	7	8	9	10	11	12	
LP variant														
SN	-[c]	2[d]	13	41	11	-	-	-	-	-	-	-	-	3.0
SNCys	-	-	-	6	21	64	8	-	-	-	-	-	-	4.7
SNLAH	-	-	-	-	-	-	-	5	36	14	8	-	-	8.4
SA	-	-	-	-	-	-	-	-	3	15	29	5	-	9.6
MP variant														
SN	-	21	50	6	-	-	-	-	-	-	-	-	-	1.8
SNCys	-	6	-	17	38	1	-	-	-	-	-	-	-	3.5
SNLAH	-	-	3	1	-	39	18	-	-	-	-	-	-	5.0
SA	-	-	-	-	-	-	-	-	1	3	13	38	25	11.0
SP variant														
SN	30	46	-	-	-	-	-	-	-	-	-	-	-	0.8
SNCys	2	77	4	-	-	-	-	-	-	-	-	-	-	1.1
SNLAH	-	8	64	6	-	-	-	-	-	-	-	-	-	2.0
SA	-	-	-	6	131	61	31	-	-	-	-	-	-	4.5

[a]Abbreviations are as follows: SN = Noble agar, SNCys = Noble agar and cysteine, SNLAH = Noble agar and lactalbumin hydrolysate, SA = agarose; all modifications of the basal skim milk medium.

[b]Mean plaque diameter (mm.).

[c]None observed.

[d]Number of plaques.

Table 16 Changes in plaque diameter resulting from the addition of bovine serum to Noble agar or agarose overlays

Overlay[a]	Plaque diameter (mm.)												Mean[b]
	0.5	1	2	3	4	5	6	7	8	9	10	11	
LP variant													
N (-)	-[c]	-	-	-	-	2[d]	4	16	1	-	-	-	6.7
N (S)	-	-	-	-	-	-	3	14	16	5	-	-	7.6
A (-)	-	-	-	-	-	-	-	2	3	19	3	-	8.9
A (S)	-	-	-	-	-	3	6	6	7	2	-	-	7.0
MP variant													
N (-)	-	-	-	-	9	33	18	-	-	-	-	-	5.2
N (S)	-	-	1	12	22	14	-	-	-	-	-	-	4.0
A (-)	-	-	-	-	-	-	-	-	6	21	30	4	9.5
A (S)	-	-	-	-	-	-	-	2	7	27	4	-	8.8
SP variant													
N (-)	11	29	36	3	-	-	-	-	-	-	-	-	1.5
N (S)	5	23	67	10	-	-	-	-	-	-	-	-	1.8
A (-)	-	-	3	4	13	56	-	-	-	-	-	-	4.6
A (S)	-	-	3	3	7	83	-	-	-	-	-	-	4.7

[a]Abbreviations indicate the following: N = Noble agar (S) with, or (-) without serum: A = agarose (S) with, or (-) without serum.

[b]Mean plaque diameter (mm.).

[c]None observed.

[d]Number of plaques.

Table 17. Neutralization of the LP Records variant by an anti-LP serum from chickens, and by three arbovirus typing sera

Antiserum[a] dilution	Plaques counted	PFU[b] neutralized	Percent[c] neutralization	End-point[d]
Anti-LP serum				
1.3	8	205	99	
2.0	39	174	90	
2.7	26	187	77	------ 3.04
3.4	151	62	22	
Anti-WEE serum				
1.3	1	212	100	
2.0	67	146	78	------ 2.44
2.7	137	76	33	
3.4	188	25	6	
Anti-EEE serum				
1.3	197	16	8	------<1.30
2.0	287	-74	0	
2.7	294	-81	0	
3.4	280	-67	0	
Anti-SLE serum				
1.3	115	98	46	------<1.30
2.0	275	-62	0	
2.7	280	-67	0	
3.4	280	-67	0	
Control serum				
1.3	193			
2.0	215			
2.7	208	Average count 213		------ 0.00
3.4	239			

[a] Negative Log_{10} .

[b] Average control count minus plaques counted.

[c] Calculated according to Reed and Muench (49).

[d] Negative Log_{10} fifty percent endpoint.

Table 18. Neutralization of the MP Records variant by an anti-LP serum from chickens, and by three arbovirus typing sera

Antiserum[a] diltuion	Plaques counted	PFU[b] neutralized	Percent[c] neutralization		End-point[d]
Anti-LP serum					
1.3	0	99	100		
2.0	0	99	100		
2.7	11	88	93		
3.4	39	60	55	------>	3.40
Anti-WEE serum					
1.3	0	99	100		
2.0	0	99	100		
2.7	11	88	93		
3.4	33	66	67	------>	3.40
Anti-EEE serum					
1.3	18	81	88	------	1.80
2.0	77	22	34		
2.7	89	10	13		
3.4	84	15	6		
Anti-SLE serum					
1.3	0	99	100	------	1.81
2.0	78	21	32		
2.7	93	6	8		
3.4	93	6	3		
Control serum					
1.3	74				
2.0	90	Average count	99	------	0.00
2.7	116				
3.4	117				

[a]Negative Log_{10} .

[b]Average control count minus plaques counted.

[c]Calculated according to Reed and Muench (49).

[d]Negative Log_{10} fifty percent endpoint.

Table 19. Neutralization of the SP Records variant by an anti-LP serum from chickens, and by three arbovirus typing sera

Antiserum[a] dilution	Plaques counted	PFU[b] neutralized	Percent[c] neutralization		End-point[d]
Anti-LP serum					
1.3	0	255	100		
2.0	23	232	96		
2.7	44	211	81	------	3.06
3.4	187	68	21		
Anti-WEE serum					
1.3	0	255	100		
2.0	40	215	89	------	2.54
2.7	145	110	38		
3.4	251	4	9		
Anti-EEE serum					
1.3	12	243	95	------	1.58
2.0	184	71	0		
2.7	289	-34	0		
3.4	323	-68	0		
Anti-SLE serum					
1.3	0	255	100	------	1.58
2.0	198	97	38		
2.7	297	-42	0		
3.4	310	-55	0		
Control serum					
1.3	153				
2.0	301	Average count	255	------	0.00
2.7	280				
3.4	287				

[a] Negative Log_{10}.

[b] Average control count minus plaques counted.

[c] Calculated according to Reed and Muench (49).

[d] Negative Log_{10} fifty percent endpoint.

Table 20. Neutralization of the LP Records variant by guinea pig antisera (averaged data from two replicate trials)

Antiserum[a] dilution	Plaques counted	PFU[b] neutralized	Percent[c] neutralization		End-point[d]
Anti-LP serum					
1.3	0	44	100		
2.0	3	41	97		
2.7	2	42	95		
3.4	14	30	71	------	3.72
4.1	28	16	25		
Anti-MP serum					
1.3	0	44	100		
2.0	3	41	96		
2.7	16	28	61	------	2.84
3.4	38	6	4		
4.1	48	-4	0		
Anti-SP serum					
1.3	3	41	97		
2.0	6	38	88		
2.7	29	15	42	------	2.58
3.4	34	10	15		
4.1	41	3	3		
Control serum					
1.3	36				
2.0	44				
2.7	43	Average count 44		------	0.00
3.4	44				
4.1	52				

[a]Negative Log_{10}.

[b]Average control count minus plaques counted.

[c]Calculated according to Reed and Muench (49).

[d]Negative Log_{10} fifty percent endpoint.

Table 21. Neutralization of the MP Records variant by guinea pig antisera (averaged data from two replicate trials)

Antiserum[a] dilution	Plaques counted	PFU[b] neutralized	Percent[c] neutralization		End-point[d]
Anti-LP serum					
1.3	0	30	100		
2.0	4	26	95		
2.7	4	26	86	------	3.38
3.4	14	16	49		
4.1	25	5	10		
Anti-MP serum					
1.3	0	30	100		
2.0	2	28	97		
2.7	8	22	80	------	3.27
3.4	14	16	43		
4.1	28	2	4		
Anti-SP serum					
1.3	4	26	90	------	1.84
2.0	12	18	30		
2.7	22	8	0		
3.4	37	-7	0		
4.1	42	-12	0		
Control serum					
1.3	26				
2.0	28				
2.7	34	Average count	30	------	0.00
3.4	30				
4.1	32				

[a] Negative Log_{10}.

[b] Average control minus plaques counted.

[c] Calculated according to Reed and Muench (49).

[d] Negative Log_{10} fifty percent endpoint.

Table 22. Neutralization of the SP Records variant by guinea pig antisera (averaged data from two replicate trials)

Antiserum[a] dilution	Plaques counted	PFU[b] neutralizes	Percent[c] neutralization		End-point[d]
Anti-LP serum					
1.3	0	64	100		
2.0	3	61	98		
2.7	23	41	74	------	3.09
3.4	46	18	31		
4.1	49	15	11		
Anti-MP serum					
1.3	0	64	100		
2.0	15	49	86	------	2.64
2.7	36	28	47		
3.4	44	20	15		
4.1	67	-3	0		
Anti-SP serum					
1.3	0	64	100		
2.0	3	61	98		
2.7	19	45	84		
3.4	22	42	62	------	3.64
4.1	35	29	27		
Control serum					
1.3	65				
2.0	50				
2.7	75	Average count	64	------	0.00
3.4	68				
4.1	61				

[a] Negative Log_{10}.

[b] Average control count minus plaques counted.

[c] Calculated according to Reed and Muench (49).

[d] Negative Log_{10} fifty percent endpoint.